Structure and Evolution of the Y-chromosomal and Mitochondrial DNA of cattle

Edward Louis Christian Verkaar

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STRUCTURE AND EVOLUTION OF THE Y-CHROMOSOMAL AND MITOCHONDRIAL DNA OF CATTLE

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(met een samenvatting in het Nederlands)

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Contents

CHAPTER 1

General introduction

The bovine Y-chromosome: structure, evolutionary context and marker of paternal origin

E.L.C.Verkaar and J.A.Lenstra

The Y-chromosome is like a bad boy: it carries the burden of only a few proper genes and goes its own way; it changes regularly its appearance, indulges in fashionable self-mutilation; always gets into trouble and refuses advice of well-meaning colleagues. It is not to be relied upon, but it knows a lot of interesting stories. How dull life would be without the Y-chromosome!

General introduction

1. Introduction

Masculinity in many species starts at the chromosomal level. Mammals, males and females share the autosomal chromosomes (22 in man, 29 in cattle) and one copy of the X-chromosome. Females have a second X-chromosome and males a single Y-chromosome. From the estimated 30,000 genes of the human haploid genome, only few are located on the Y-chromosome. One of these is the *SRY* (sex reversal Y-encoded) gene, which directly governs the gender. Several other Y-chromosomal genes are involved in spermatogenesis. The remaining of the Y-chromosome consists for a large part of repetitive DNA (Kuroda-Kawaguchi *et al.* 2001; Skaletsky *et al.* 2003; Tilford *et al.* 2001b) and is presumably without any function. In contrast to the Y chromosome, the X-chromosome is a large chromosome with a high gene content. Recent studies showed that the sex chromosomes descend from a pair of homologous autosomes and that their similarity disappeared by specialization and degeneration of the Y-chromosome.

In this chapter we describe the anatomy of the human and bovine Y-chromosomes, the unique mode of evolution of the Y-chromosome, the application of Y-chromosomal markers as probes of the origin of tissue samples and population history or species formation. In this context we then present an outline of this thesis.

2. Anatomy of the human and cattle Y-chromosomes

A comparison of the human and mouse genomes (Mouse Genome Sequencing Consortium, Lander *et al.* 2001) has indicated that about 40% of the autosomal and X-chromosomal sequences and 99% of the genes have been retained during the mammalian evolution. However, the mammalian Y-chromosome seems to be much more variable (Hellborg and Ellegren 2003; Tilford *et al.* 2001a; Wyckoff *et al.* 2002). Nevertheless, several genes and functional properties are probably shared by most mammalian Y-chromosomes (Hellborg and Ellegren 2003). In this section we describe the Y-chromosome of men as model for the

mammalian Y-chromosome and then summarize the available literature on the Y-chromosome of cattle.

2.1 The human Y-chromosome

The divergence of the mammalian sex chromosomes has limited their meiotic recombination to the pseudoautosomal (PAR) regions at one or both chromosomal ends. The human Y-chromosome (Skaletsky *et al.* 2003) has on the short arm (Yp) and long arm (Yq) PAR regions of 2600 and 500 kb, respectively. The remainder of the Y-chromosome is designated as MSY (male-specific part of Y-chromosome, until recently denoted as NRY). It is 63 Mb in size and contains 23 Mb euchromatic MSY and a heterochromatic block spanning most of Yq. The heterochromatin is devoid of genes and may function as a stabilizer of the Y-chromosome (Marshall Graves 2000).

The best characterized Y-chromosomal gene is *SRY*, which is present in several mammals (Margarit *et al.* 1998). Deletion of the gene in XY genotypes or insertion in XX genotypes causes sex-reversal. Fourteen human Y-chromosomal genes are located in the PAR's and have an X-chromosomal counterpart (Ciccodicola *et al.* 2000; Graves *et al.* 1998a). In addition, the human MSY contains a segment copied from the X-chromosomal Xq21 region after the human-chimpanzee divergence. The other genes on the human MSY can be divided into two classes (Lahn and Page 1997; Quintana-Murci *et al.* 2001). In the first class are 18 so-called 'X-degenerate' genes, which are present in single-copy, have a X-chromosomal homologue and, with the exception of the tooth-developing gene *AMELY*, are ubiquitously expressed housekeeping genes. *SRY* can also be classified as X-degenerate and is distantly related to the X-chromosomal gene *SOX3* (Marshall Graves 2000). The second class comprises 63 'ampliconic' multicopy Y-chromosomal genes, which belong to nine gene families and are expressed in the testis only. With the exception of *RBMY* and *VCY* genes, these genes have no X-chromosomal homologue. Several of these genes are located in one of eight palindromic regions, which occupy 25 % of the MSY euchromatin (Lahn and Page 1997; Quintana-Murci *et al.* 2001). Gene copies on the two arms of each palindrome have more than 99.9% identity as a result of fast gene conversions (Rozen *et al.* 2003). This horizontal recombination mechanism acts for the MSY as substitute for the interallellic recombination with sister chromosomes. Next to the protein-coding genes, the

human MSY contains 75 putative non-coding transcription units (Lahn and Page 1997; Quintana-Murci *et al.* 2001).

TSPY (testis-specific protein Y-encoded) is the only ampliconic gene outside the palindromic regions. This gene has 35 copies, which are all contained within a 20-kb unit of the *DYZ-5* tandem repeat (Dechend *et al.* 2000; Vogel *et al.* 1998a) on Yp near the centromere (Lahn and Page 1997; Quintana-Murci *et al.* 2001). The *TSPY* protein belongs to the TTSN (*TSPY*, *TSPY*-like, *SET*, *NAP-1*) superfamily and is involved in early spermatogenesis (Vogel *et al.* 1998b). It is also a candidate for *GBY* (gonadoblastoma-inducing gene, Y-encoded; acting in XY females) and is suspected to act as an oncogene in testicular tumors (Lau 1999; Lau *et al.* 2000; Lau and Zhang 2000). So far, *TSPY* genes have been identified in several primates, rodents, artiodactyls (Vogel and Schmidtke 1998) and horse (Manz *et al.* 1993). However, *TSPY* is not a multicopy gene in all mammals. The rat Y-chromosome contains only one intact copy (Dechend *et al.* 1998) and the mouse Y-chromosome carries only a single pseudogene (Schubert *et al.* 2000).

The mode of action of the Y-chromosomal genes is only partially understood. Several encoded proteins act as transcription factors (Cotinot *et al.* 2002; Veitia *et al.* 2001) or RNA-binding proteins (Quintana-Murci *et al.* 2001). *SRY* contains a DNA-binding HMG (high-mobility group) box and activates the genes *SOX9* and *SF1*. This triggers the development of the testes, but this does not imply that the development of females is a default pathway (Cotinot *et al.* 2002; Mackay 2000). The action of *SRY* is counteracted by an extra copy of the X-chromosomal gene *DAX1* in XY females with a duplication on the X-chromosome (Morrish and Sinclair 2002). *TSPY* encodes a nucleosome-binding protein (Vogel and Schmidtke 1998).

Not surprisingly, major fertility traits as azoospermia, sex-reversal and hermaphroditism are associated with Y-chromosomal deviations (Ali and Hasnain 2002; Cotinot *et al.* 2002; Jobling and Tyler-Smith 2000; Kuroda-Kawaguchi *et al.* 2001; Oates *et al.* 2002; Queipo *et al.* 2002; Quintana-Murci *et al.* 2001). Molecular variation on the Y-chromosome has been correlated with spermatogenesis, growth and aggressiveness (Quintana-Murci *et al.* 2001; Jobling and Tyler-Smith 2000). In this respect, it may be hypothesized that the artificial selection that accompanied the domestication of livestock species included selective sweeps and the fixation of more ‘tame’ Y-chromosomes.

2.2 The bovine Y-chromosome

The Y-chromosomes of taurine cattle (*Bos taurus*) and of the related bovine species gaur (*Bos gaurus*) and banteng (*Bos javanicus*) have a metacentric morphology and are in metaphysic spreads readily distinguishable from the acrocentric autosomes. However, this is not a typical feature as the Y-chromosome of zebu (*Bos indicus*) is acrocentric (Goldammer *et al.* 1997) as was probably the morphology of the *Bovini* ancestral Y-chromosome (Gallagher *et al.* 1999). Like in men, morphological abnormalities of the bovine Y-chromosome have been associated with a female phenotype (Iannuzzi *et al.* 2001a) or azoospermia (Iannuzzi *et al.* 2001b).

At present, not much is known about the gene content of the bovine Y-chromosome. Bovine *SRY* (Daneau *et al.* 1995) has been localized in the Yq region (Iannuzzi *et al.* 2001b). Two other X-degenerate single copy genes have been identified, *AMELY* (amelogenin, Y-encoded: (Gibson *et al.* 1991) and *ZFY* (zinc finger protein, Y-encoded: (Xiao *et al.* 1998). The ampliconic *TSPY* gene (Jakubiczka *et al.* 1993; Vogel *et al.* 1997a; Vogel *et al.* 1997b) is reported to exist in 50 to 200 copies dispersed over the bovine MSY. Our data presented in Chapter 4 lead to an estimate of 90 loci, most of which are on Yp. About half of the loci contain a functional gene and the other half a cluster of at least 4 truncated pseudogenes.

In another artiodactyl species, the pig, radiation hybrid mapping of the Y-chromosome revealed a gene order resembling the murine Y-chromosomal genes: *Ypter*-(*AMELY-EIF2S3Y/ZFY-USP9Y-DBY/UTY*)-(*TSPY-SMCY-UBE1Y-SRY*)-*CEN* (Quilter *et al.* 2002). Except *TSPY*, these are all X-degenerate genes, but *UBE1Y* and *EIF2S3Y* are not present in humans. Since the porcine X homologues had the same gene order, it is proposed that this also resembles the gene order on the ancestral eutherian sex chromosome.

Bovine Y-chromosomal repetitive elements have attracted commercial interest because of their potential use as probes for the sexing of embryos (Miller and Koopman 1990). For the following sequences the male-specificity and estimated copy number are in the public domain:

- BC 1.2, a 54 bp motif present in 2000 to 2500 copies (Cotinot *et al.* 1991; Popescu *et al.* 1988), located on Yp13-p12 and related to λES 6.0 (Bondioli *et al.* 1989; Schwerin *et al.* 1992).

- BRY.1, a 307-bp *Sau*3A1 fragment and dispersed over the entire Y-chromosome (Schwerin *et al.* 1992; Thomsen and Jorgensen 1994). BRY.1 is probably part of a larger Y-chromosomal sequence. It is imbedded within a BRY.2 sequence (Matthews and Reed 1992), which is only partially male-specific. Another part of BRY.2 is similar to exon 1a to exon 3 of *TSPY* (see chapter 4).
- BtDYZ1, a tandem repeat of 60 units consisting of a 40-bp motif and a TG-rich sequence of 12 to 63 bp. This assembly is present in 60.000 copies, which corresponds to 6% of the Y-chromosome. BtDYZ1 is localized in the pericentric region (Perret *et al.* 1990).

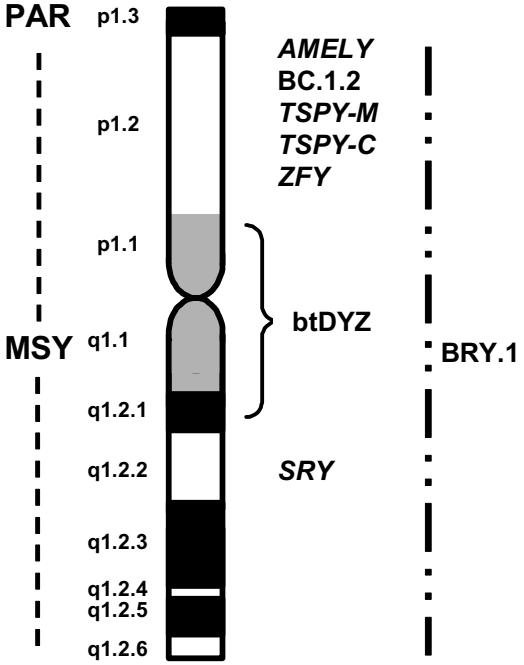

Figure 1. Physical map of the bovine Y-chromosome.

Microsatellites in the bovine PAR have been mapped by linkage analysis (Sonstegard *et al.* 2001). Several MSY microsatellites, that were not multicopy could be localized by radiation hybrid mapping (Liu *et al.* 2002). The multicopy marker IDVGA50 has been localized by fluorescent *in situ* hybridization in the Yq region (Iannuzzi *et al.* 2001b) and is associated with the *TSPY* loci (chapter 4).

Fig. 1 summarizes the known cytogenetic localizations of bovine Y-chromosomal sequences.

3. Evolution of the Y-chromosome

The dynamic evolution of the mammalian Y-chromosome is evident from its divergence of the X-chromosome, from major differences between Y-chromosomes of different mammalian species and also from the prevalence of male infertility associated with Y-chromosomal deletions. Theoretical considerations as well as experimental observations have made plausible that the evolution of the Y-chromosome is driven by several processes that are not, or much less, relevant for the evolution of the autosomes and the X-chromosome:

- Genetic hitch-hiking and other processes leading to an accumulation of mutations due to a lack of recombination (Charlesworth and Charlesworth 2000).
- The occurrence of additions and attritions of genes involved in masculine properties or male fertility.
- The continuous state of hemizygosity that emphasizes the involvement of Y-chromosomal genes in the control of male properties.
- The involvement in genetic conflicts that continuously selects new variants to compensate for mutations in antagonistic loci.

These processes are discussed in more detail below.

3.1 Lack of recombination

The MSY is the only region of the nuclear genome that does not recombine during meiosis. This eliminates a mechanism for the removal of deleterious mutations. In the Y-chromosome of the higher primates mutations in ampliconic genes in the palindromic regions

are removed by gene conversions (Rozen *et al.* 2003). However, these palindromes do not contain all MSY genes, while nothing is known about palindromes on the Y-chromosomes of other species. The lack of recombination leads to degeneration via various mechanisms: A stochastic loss of haplotypes with the fewest mutations by genetic drift is known as *Muller's ratchet* (Charlesworth and Charlesworth 2000). *Genetic hitchhiking,* occurs when fixation of a beneficial mutation leads to the fixation of another deleterious mutation. Other related processes are *background selection* and the *weak Hill-Robertson* effects (Charlesworth and Charlesworth 2000).

We note that all these four mechanisms are sofar hypothetical. Theoretical simulations (Charlesworth and Charlesworth 2000) did not indicate which of these mechanisms contributed most to the loss over time of functionality and decreasing diversity of the Y-chromosome (Ali and Hasnain 2002; Graves and Delbridge 2001; Marshall Graves 2000; Quintana-Murci *et al.* 2001). In view of the selective forces acting on the Y-chromosome (see below), a predominant role of genetic hitch-hiking seems most plausible (Graves *et al.* 1998a; Mitchell *et al.* 1998; O'Neill *et al.* 2001; Rice and Holland 1997).

3.2 Addition, attrition and functionality of Y

The X and Y chromosomes descend from a pair of homologous autosomes (Graves *et al.* 1998b). In several publications (Graves 2002; Marshall Graves 2000; Mitchell *et al.* 1998; Waters *et al.* 2001) this evolution is described as a continuing process of adding autosomal segments (addition) to and the subsequent degradation of these constituents of the Y-chromosome (attrition). Evidence for this scenario is based on comparison of the gene contents of marsupials and placental mammals (Toder *et al.* 2000). The regions of the X- and Y-chromosomes that originate from the proto-X-Y chromosomes are called XCR and YCR (X and Y conserved region), respectively. The YCR now only comprises a small fraction of the MSY and contains the X-degenerate genes *SRY*, *RBMY*, *RPS4Y* and *SMCY*. These genes are also present on the Y-chromosome of marsupials (Waters *et al.* 2001). Other X-degenerate genes on the MSY are derived from a later addition, 80 to 130 million years ago, of an autosomal segment to the X and Y-chromosomes, called XAR and YAR (X and Y added regions), respectively. The XAR/YAR genes are localized on the autosomal 5p region of the marsupial wallaby, suggesting that these genes were part of a single addition to the PAR

region of the eutherian sex-chromosomes, 80-130 million years ago (Toder *et al.* 2000; Waters *et al.* 2001).

A more detailed picture has been presented by Lahn and Page (Lahn and Page 1999b) and Skaletsky *et al.* (2003), who compared the Y-chromosomal X-degenerate genes with their X-homologues and, on the basis of the X-Y divergence, differentiated four groups. Groups 1 and 2 are in the YCR and groups 3 and 4 in the YAR. The divergence with the X-homologues is proposed to reflect the time period of inversion events (Lahn and Page 1999b), which moved the genes from the PAR to the MSY and stopped the recombination of the X- and Y-chromosomal homologues. A clear correlation between the divergence of the X-Y divergence and the gene order of the X-homologues (Skaletsky *et al.* 2003) indicates that this was a gradual process, which took place from 320 Myr ago (the age of the primitive mammals) to 30 Myr ago (after the divergence of the first primates). Insertions of autosomal genes in the MSY regions generated the ampliconic genes (Gromoll *et al.* 1999; Lahn and Page 1997; Lahn and Page 1999a). With the exception of TSPY, these genes became part of palindromic regions (Skaletsky *et al.* 2003). Most Y-chromosomal genes were deleted or degenerated (Marshall Graves 2000). The surviving genes acquired male-specific functions, a process that probably has been accelerated by the hemizygosity of the sex chromosomes (see below).

It has been proposed that continuous degeneration of MSY will ultimately lead to the loss of the Y-chromosome (Graves 2002). Extinction has already occurred in the vole mole in which the gender is controlled by the number of X-chromosomes (XX, females;XO, males). Marshall Graves (2002) predicted an extinction of the mammalian Y-chromosome within 10 to 100 Myr.

3.3 The continuous state of hemizygosity

Recently, it was found that relatively many genes essential for spermatogenesis were localized on the X-chromosome (Graves and Delbridge 2001; Hurst 2001; Wang *et al.* 2002). This was explained by a clever reasoning already put forward by Rice (Rice 1987). In a way of thinking that is typical in evolutionary biology, it is considered what happens if a mutation in a X-chromosomal gene is favorable in males, but neutral or slightly deleterious in heterozygous females. For instance, a change in a translation initiation factor may have a

favorable effect on the viability of sperm cells. This mutation will be rapidly selected in males, which are hemizygous (i.e., having one gene variant by having only one gene copy rather than by being homozygous), and spread among the population. When the mutation gains a higher frequency, homozygous females are born with possibly a lower fitness. However, as soon as a mutation in another gene compensates for this unfavorable effect, the X-chromosomal mutation is likely to become fixed because of the continuing positive selection in males. As a result, the X-chromosomal genes have become especially useful for the sperm cells.

In contrast, a mutation with a similar effect on the sperm cells on one of the autosomes would be heterozygous in both males and females and the selection in males would be weaker. Thus hemizygosity in males acts as a filter that retains those alleles favorable in males.

It is generally overlooked in the molecular genetic literature that this effect of hemizygosity is much stronger for the Y-chromosome than for the X-chromosome. In the first place, Y-chromosomal variants are always hemizygous, while X-chromosomal variants are hemizygous only in one-third of the time. Secondly, no mutations in other genes have to be postulated in order to compensate for any negative effect in females. We hypothesize that the hemizygous state of the Y-chromosome drives an accumulation of male-benefit alleles (Rice and Holland 1997). As a consequence, Y-chromosomal genes are recruited for functions related to male properties, which contributes to the divergence of the sex chromosomes. In other words, the involvement of Y-chromosomal genes in male development and spermatogenesis may be caused by selection of male-benefit *alleles* rather than accumulation of male-benefit *genes* (Lahn and Page 1997).

3.4 Genetic conflicts

Genetic conflicts arise if selection has opposite effects on different loci and leads to a antagonistic co-evolution (Rice and Holland 1997). If the conflict is between loci of one species, this process is denoted as *interlocus contest evolution* (ICE). Several categories of genetic conflicts have been observed in a variety of species (Hurst 2001; Partridge and Hurst 1998). We mention two conflicts in which the Y-chromosome may become involved:

1. Sperm cells and oocytes have conflicting interests during fertilization. Sperm cells with mutations that allow a better penetration of oocytes will have a selective advantage. However, if this leads to the entry of more than one sperm cell it is not in the interest of the oocyte. Genes expressed on the oocyte surface then will try to dampen the enthusiasm of the sperm cells via mutations affecting surface proteins.
2. *Meiotic drive* (Hurst 1998; Pomiankowski and Hurst 1993) is the fight of two autosomal alleles or sex chromosomes for a place in the haploid germ cell. For an X-chromosome it would be advantageous if sperm cells that carry a Y-chromosome would be killed and vice versa. For example, a mutant X-chromosomal protein may, after meiosis, bind to a Y-chromosomal protein and then become harmful. Only X-containing germ cells survive and the mutant X-chromosome will spread at the expense of the Y-chromosome as well as X-chromosomes without the mutation. It has been proposed (Amos and Harwood 1998) that in this conflict the X-chromosomes are prevailing because of their numerical superiority and that the Y-chromosome is forced to inactivate its genes it order to prevent recognition by X-chromosomal expression products. However, any effect of meiotic drive on the male-female ratio is counteracted by a feedback on the sex-ratio (Ridley 1996). As soon as there are less than 50% males, male individuals will on the average have more than one partner and transmit more of their genetic material to the next generation than female individuals. So it becomes advantageous to have male rather than female offspring, either by reversing the effect of the mutations that has started the meiotic drive or by an antagonistic mutation in another locus.

The effects of genetic conflicts and the maintenance of the sex ratio are often described as *Red Queen* adaptation. Like the Red Queen is 'running just to keep in the same place' (Carrol 1887), a locus is adapting to respond to a change in another locus and any advantage gained is only temporary (Ridley 1994). Although convincing evidence has been presented for the existence of genetic conflicts in several organisms (Hurst *et al.* 1996), this does not imply that the above described conflicts always exist: the mutations generating the conflict may just happen or not. The molecular mechanisms behind the conflicts are not

always specified and are left to our imagination. However, the maintenance of the sex ratio is more than hypothetical. Thus it is safe to assume that gene variants exist that favor the birth of either males of female. Because of the function of the Y-chromosomal genes, it is also plausible that the Y-chromosome is one of the most obvious targets for any effects of sex-related genetic conflicts (Rice and Holland 1997).

3.5 Effects on the evolution of the Y-chromosome

Although more work is needed to verify the consequences of a lack of meiotic recombination, additions and attritions, hemizygosity and genetic conflicts, respectively, their postulated effects are entirely consistent with well-documented characteristics of the Y-chromosome:

1. A high degree of divergence of the sex-chromosomes since their common descendance from a pair of homologous autosomes (Graves 2002; Lahn and Page 1999a; Marshall Graves 2000; Waters *et al.* 2001; Wyckoff *et al.* 2002).
2. A rapid and punctuated evolution via *genetic sweeps* (Aitken and Marshall Graves 2002; Amos and Harwood 1998; Quintana-Murci *et al.* 2001). This is illustrated by the inversions within the Y-chromosome during the divergence of the bovine species (Gallagher *et al.* 1999), by the erratic evolution of the primate *SRY* (Wang *et al.* 2001) and by the observation that transgenic introduction of *SRY* in an XX mouse does not induce the formation of external genitalia if the *SRY* has been derived from another mouse strain (Coward *et al.* 1994).
3. A continuing degeneration with few active genes and many pseudogenes (Lahn *et al.* 2001; Marshall Graves 2000; Rice and Holland 1997). In fact, this degeneration also manifests itself on the human population level by deletions that cause male infertility (Ali and Hasnain 2002; Jobling *et al.* 1998a; Quintana-Murci *et al.* 2001).

According to a new insight (Skaletsky *et al.* 2003) the ampliconic genes located in the palindromic region of the human Y-chromosome maintain their integrity by gene conversion. In this way, this horizontal recombination protects a part of the Y-chromosomal genes against degeneration. In chapter 4 we conclude on the basis of the species distribution of *TSPY* sequence variants that the bovine *TSPY* genes are also subject to a rapid horizontal evolution.

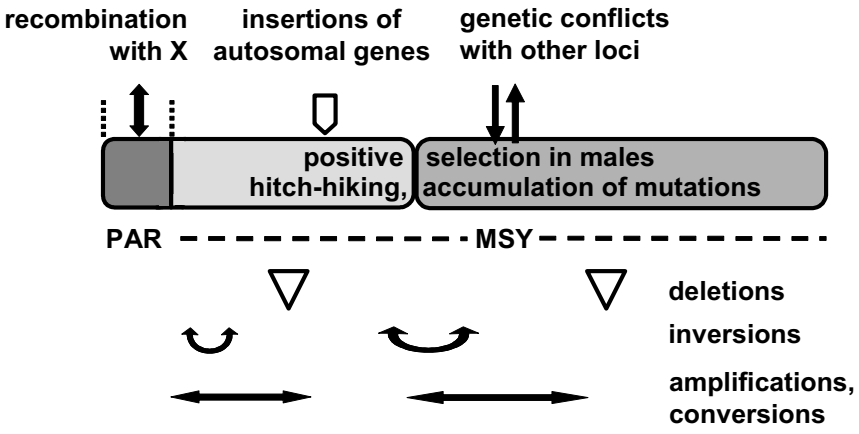

Figure 2. Genetic interactions driving the evolution of the Y-chromosome.

Figure 2 summarizes the genetic interactions that shape the dynamic evolution of the Y-chromosome.

4. Markers for descendance and species origin; Y-chromosomal variation

The nuclear and mitochondrial genomes are an invaluable source of markers to trace the origin of individuals, populations or species (Nijman *et al.* 1999). The choice of the most suitable marker depends on the mode of inheritance (maternal, paternal or both) as well as the time window in which it must be informative. For instance, markers with a high mutation rate are useful for genetic studies, but are less suitable for species comparison because of divergence saturation and/or their absence in other species. In this section, we describe the various categories of human and bovine markers for genetic and evolutionary studies and in this context discuss the use of Y-chromosomal markers as landmarks for paternal lineages.

4.1 Microsatellites and minisatellites

Tandem repeats of 2 to 5 bp (microsatellites or SSRs, simple-sequence repeats) or up to 30 bp (minisatellites or VNTRs, variable number of tandem repeats) are used extensively for linkage studies, paternity testing, identity-by-descent mapping and genetic diversity studies in men, mice and domestic animals. For instance, bovine microsatellites have been used to study African pastoralism (Hanotte *et al.* 2000) and the introgression of taurine cattle in Asian zebu breeds. However, these markers are generally less suitable for phylogenetic studies on the species level (Ritz *et al.* 2000).

4.2 Mitochondrial DNA

Mitochondrial DNA (mtDNA) has relatively conserved and more variable regions, which both can be used for comparison of mammalian orders, species as well as populations. For closely related species it is also relevant that mitochondrial markers are only transmitted via the maternal lineage. Studies of human (mtDNA) has provided evidence for the out-of-Africa hypothesis, which states that all human individuals descend via the maternal lineage from one woman that lived in Africa about 150,000 years ago (Ingman *et al.* 2000; Templeton 2002). It is also allowed ? conclusions about relationships of human populations (Cavalli-Sforza and Minch 1997; Templeton 2002) and provided evidence that the extinct Neanderthals were genetically isolated from modern *Homo sapiens* (Krings *et al.* 1997). However, most human mitochondrial DNA variants have now a rather broad geographical distribution (Oota *et al.* 2001).

Lack of population specificity is also observed for mitochondrial variants of cattle (Bradley *et al.* 1996). It has been demonstrated that European taurine cattle originates from the Middle East (Troy *et al.* 2001) and that Southern-European cattle has been subject to introgression of African taurine breeds (Cymbron *et al.* 1999). Historically it is of interest that taurine cattle (*Bos taurus*) and zebu (*Bos indicus*) are the result from separate domestications and that African zebus descend maternally from taurine herds (Cymbron *et al.* 1999). Anomalous mitochondrial lineages have also been found in other bovine populations:

- Taurine mitochondria in American bisons (Polziehn *et al.* 1995; Ward *et al.* 1999), Chapter 3).

- Banteng mitochondria in Indonesian cattle (Nijman *et al.* 2002a).
- Zebu mitochondria in the Malaysian population of Bali cattle that is supposed to be a domestic form of the banteng (Nijman *et al.* 2002a).
- Zebu mitochondria in a yak individual (Janecek et al., 1996; Ward et al., 1999; chapter 3 of this thesis)
- Yak mitochondria in South-Chinese cattle (Yu *et al.* 1999) and Nepal cattle (Kikkawa *et al.* 2003).

At the species level most reconstructions of phylogenetic trees have so far relied on mitochondrial DNA. This has led to partially resolved phylogenies of the family of the *Bovidae* (Gatesy *et al.* 1997; Hassanin and Douzery 1999) and the tribe of the *Bovini* (Janecek *et al.* 1996). For the *Bovini*, or cattle species, these studies are complicated by anomalies in the maternal lineage (Verkaar *et al.* 2002a). In Chapter 3 we present an almost complete resolution of the maternal phylogeny of the bovine species. We observed close relationships of American bison *(Bison bison*) and yak (*Bos grunniens*), and of banteng (*Bos javanicus*), gaur (*Bos gaurus*) and gayal (*Bos frontalis*). The European bison (wisent, *Bison bonasus*) clearly occupies a position separate from the American bison.

4.3 Point mutations in nuclear DNA

The human genome project has identified many SNP's (single nucleotide polymorphisms) and these markers are predicted to succeed the microsatellites as genetic markers (Gray *et al.* 2000). However, since nuclear DNA mutates slower than mitochondrial DNA (Chikuni *et al.* 1995), correspondingly more data has to be collected in order to obtain the same resolving power in phylogenetic studies. Studies on *Bovidae* (Matthee and Davis 2001) and artiodactyls (Matthee *et al.* 2001) were complementary to the analysis of mitochondrial DNA (Gatesy *et al.* 1997; Hassanin and Douzery 1999). AFLP fingerprinting (Buntjer *et al.* 2002) is also based on nuclear DNA variation and indicated genetic exchange between *Bovini* species. This has been substantiated by comparison of the maternal and paternal lineages within this tribe (see below).

4.4 SINE insertions

SINE retrotranspositions (Deininger and Batzer 2002) are likely to be UME (unique mutational events) and have been used as markers of population history (Romualdi *et al.* 2002; Templeton 2002) or phylogeny (Nijman *et al.* 2002b; Nikaido *et al.* 1999). At a higher taxonomic level, SINEs can be used as probes for mammalian orders or suborders (Buntjer *et al.* 1997; Nikaido *et al.* 1999). In Chapter 1c we describe a quantitative PCR of the Bov-A2 SINE repeat (Lenstra *et al.* 1993) as a probe of ruminant material. This assay is useful for the inspection of feedstuff in order to prevent the spread of prion-induced diseases.

4.5 Satellite DNA

Satellite DNA is a reliable marker of the species, while satellite polymorphisms (SFLP) are used for species hybridization (Nijman *et al.* 1999). In chapter 2a we demonstrate the use of SFLP as a tool for bovine species identification as complementary to mitochondrial PCR-RFLP (Verkaar *et al.* 2002b) and particularly valuable if species introgression is suspected.

4.6 The power of Y-chromosomal markers

During the last six years markers based on Y-chromosomal variation have been introduced in human genetics (de Knijff 2000; Hurles and Jobling 2001). Y-chromosomal markers are transmitted along the paternal lineage and complement the information derived from mtDNA (Boissinot and Boursot 1997). In human genetics, earlier studies based on mtDNA and autosomal markers have been correlated with Y-chromosomal data. This has generated new evidence for the early origin of *Homo sapiens* in Africa (Gibbons 1997; Hedges 2000; Ke *et al.* 2001). In addition, studies on the colonization of the American continent, the migration of populations in Asia, Europe and several other demographic events (Amos and Harwood 1998; de Knijff 2000; Jobling *et al.* 1998b; Malaspina *et al.* 1998) have provided new insights into human phylogeography (Hurles and Jobling 2001). The discordance of paternal and maternal lineages appears to be caused by factors like unequal female-to-male migration rates and polygamy (Cavalli-Sforza and Minch 1997; Oota *et al.* 2001; Seielstad *et al.* 1998).

Y-chromosomal markers in bovine species are scarce. Several Y-chromosomal microsatellites have been isolated, but most are in the PAR region, occur in several copies or are monomorphic (Liu *et al.* 2002; Van Hooft *et al.* 2002). Edwards *et al.* (2000) collected data on the alleles of four microsatellites in different bovine species and observed polymorphisms within taurine populations for marker INRA126. Microsatellites have been used for the differentiation of Y-chromosomes from zebu and taurine cattle (Giovambattista *et al.* 2000; Hanotte *et al.* 2000), and from bison and taurine cattle (Ward *et al.* 2001). Van Hooft (2001) observed differences among African buffalo populations with regard to the diversity of microsatellite INRA008. Ongoing efforts directed towards the typing of other markers and the identification of Y-chromosomal SNP's offer the perspective of isolating markers that are informative for the history of domestication.

We have used point mutations in the *SRY* and *ZFY* genes as markers for paternal lineages in bovine hybrid populations (Chapter 1b) and obtained evidence for male banteng introgression in the Indonesian Madura zebu breed. Several reports have used Y-chromosomal markers for comparing the paternal phylogeny with maternal trees based on mitochondrial DNA (Cathey *et al.* 1998; Kadwell *et al.* 2001; Pecon Slattery and O'Brien 1998; Tosi *et al.* 2000). In Chapter 3 we present a paternal tree of the bovine species. In this tree, American and European wisent appeared to be closely related. Since this does not agree with the divergence of the mtDNAs of these species, it is proposed that wisent has a hybrid origin and emerged by introgression of bison bulls in an ancestral bovine population related to taurine cattle. This is a new mode of speciation, for which we propose the term *transpatric* as distinct from the canonical allopatric, sympatric and parapatric modes (Ridley 1996).

5. Outline of this thesis

This thesis describes the structure and evolution of the cattle Y-chromosome in relation to more general aspects of the Y-chromosomal evolution and demonstrate the use of Y-chromosomal-, autosomal or mitochondrial variation for diagnostic assays, Chapter 2 describes the use of these markers. Autosomal variation in satellite DNA (Chapter 2a) and Y-chromosomal markers (Chapter 2b) is shown to be complementary to mitochondrial markers, while autosomal SINE elements are useful for the quantification of ruminant material in feedstuff (Chapter 2c). In chapter 3 we study the evolution of the *Bovini* tribe by comparing their Y-chromosomal *SRY* and *ZFY* genes and mitochondrial DNA sequences. In Chapter 4 we describe the *TSPY* ampliconic gene families and present evidence for concerted evolution within the Y-chromosome. The last chapter is a summarizing discussion.

References

Aitken, R. J., and J. A. Marshall Graves, 2002 The future of sex. Nature **415:** 963.

Ali, S., and S. E. Hasnain, 2002 Molecular dissection of the human Y-chromosome. Gene **283:** 1-10.

Amos, W., and J. Harwood, 1998 Factors affecting levels of genetic diversity in natural populations. Philos Trans R Soc Lond B Biol Sci **353:** 177-186.

Boissinot, S., and P. Boursot, 1997 Discordant phylogeographic patterns between the Y chromosome and mitochondrial DNA in the house mouse: selection on the Y chromosome? Genetics **146:** 1019-1034.

Bondioli, K., S. Ellis, J. Pryor, M. Williams and M. Harpold, 1989 The use of male-specific chromosomal DNA fragments to determine the sex of bovine preimplantation embryos. Theriogenology**:** 95-104.

Bradley, D. G., D. E. MacHugh, P. Cunningham and R. T. Loftus, 1996 Mitochondrial diversity and the origins of African and European cattle. Proc Natl Acad Sci U S A **93:** 5131-5135.

Buntjer, J. B., I. A. Hoff and J. A. Lenstra, 1997 Artiodactyl interspersed DNA repeats in cetacean genomes. J Mol Evol **45:** 66-69.

Buntjer, J. B., M. Otsen, I. J. Nijman, M. T. Kuiper and J. A. Lenstra, 2002 Phylogeny of bovine species based on AFLP fingerprinting. Heredity **88:** 46-51.

Carrol, L., 1887 Through the looking glass. Penguin books.

Cathey, A. C., J. W. Bickham and J. C. Patto, 1998 Introgressive hybridization and noncongruent evolutionary history of maternal and paternal lineages in north-american deer. Evolution **52:** 1224-1229.

Cavalli-Sforza, L. L., and E. Minch, 1997 Paleolithic and Neolithic lineages in the European mitochondrial gene pool. Am J Hum Genet **61:** 247-254.

Charlesworth, B., and D. Charlesworth, 2000 The degeneration of Y chromosomes. Philos Trans R Soc Lond B Biol Sci **355:** 1563-1572.

Chikuni, K., Y. Mori, T. Tabata, M. Saito, M. Monma *et al.*, 1995 Molecular phylogeny based on the kappa-casein and cytochrome b sequences in the mammalian suborder Ruminantia. J Mol Evol **41:** 859-866.

Ciccodicola, A., M. D'Esposito, T. Esposito, F. Gianfrancesco, C. Migliaccio *et al.*, 2000 Differentially regulated and evolved genes in the fully sequenced Xq/Yq pseudoautosomal region. Hum Mol Genet **9:** 395-401.

Cotinot, C., M. Kirszenbaum, M. Leonard, L. Gianquinto and M. Vaiman, 1991 Isolation of bovine Y-derived sequence: potential use in embryo sexing. Genomics **10:** 646-653.

Cotinot, C., E. Pailhoux, F. Jaubert and M. Fellous, 2002 Molecular genetics of sex determination. Semin Reprod Med **20:** 157-168.

Coward, P., K. Nagai, D. Chen, H. D. Thomas, C. M. Nagamine *et al.*, 1994 Polymorphism of a CAG trinucleotide repeat within Sry correlates with B6.YDom sex reversal. Nat Genet **6:** 245-250.

Cymbron, T., R. T. Loftus, M. I. Malheiro and D. G. Bradley, 1999 Mitochondrial sequence variation suggests an African influence in Portuguese cattle. Proc R Soc Lond B Biol Sci **266:** 597-603.

Daneau, I., A. Houde, J. F. Ethier, J. G. Lussier and D. W. Silversides, 1995 Bovine SRY gene locus: cloning and testicular expression. Biol Reprod **52:** 591-599.

de Knijff, P., 2000 Messages through bottlenecks: on the combined use of slow and fast evolving polymorphic markers on the human Y chromosome. Am J Hum Genet **67:** 1055-1061.

Dechend, F., S. Schubert, I. Nanda, T. Vogel, M. Schmid *et al.*, 1998 Organization and expression of rat Tspy. Cytogenet Cell Genet **83:** 270-274.

Dechend, F., G. Williams, B. Skawran, S. Schubert, M. Krawczak *et al.*, 2000 TSPY variants in six loci on the human Y chromosome. Cytogenet Cell Genet **91:** 67-71.

Deininger, P. L., and M. A. Batzer, 2002 Mammalian retroelements. Genome Res **12:** 1455-1465.

Edwards, C. J., C. Gaillard, D. G. Bradley and D. E. MacHugh, 2000 Y-specific microsatellite polymorphisms in a range of bovid species. Anim Genet **31:** 127-130.

Gallagher, D. S., Jr., S. K. Davis, M. De Donato, J. D. Burzlaff, J. E. Womack *et al.*, 1999 A molecular cytogenetic analysis of the tribe Bovini (Artiodactyla: Bovidae: Bovinae) with an emphasis on sex chromosome morphology and NOR distribution. Chromosome Res **7:** 481-492.

Gatesy, J., G. Amato, E. Vrba, G. Schaller and R. DeSalle, 1997 A cladistic analysis of mitochondrial ribosomal DNA from the Bovidae. Mol Phylogenet Evol **7:** 303-319.

Gibbons, A., 1997 Y chromosome shows that Adam was African [news]. Science **278:** 804-805.

Gibson, C., E. Golub, R. Herold, M. Risser, W. Ding *et al.*, 1991 Structure and expression of the bovine amelogenin gene. Biochemistry **30:** 1075-1079.

Giovambattista, G., M. V. Ripoli, J. C. De Luca, P. M. Mirol, J. P. Liron *et al.*, 2000 Male-mediated introgression of Bos indicus genes into Argentine and Bolivian Creole cattle breeds. Anim Genet **31:** 302-305.

Goldammer, T., R. M. Brunner and M. Schwerin, 1997 Comparative analysis of Y chromosome structure in Bos taurus and B. indicus by FISH using region-specific, microdissected, and locus-specific DNA probes. Cytogenet Cell Genet **77:** 238-241.

Graves, J. A., 2002 Evolution of the testis-determining gene--the rise and fall of SRY. Novartis Found Symp **244:** 86-97; discussion 97-101, 203-106, 253-107.

Graves, J. A., and M. L. Delbridge, 2001 The X--a sexy chromosome. Bioessays **23:** 1091-1094.

Graves, J. A., C. M. Disteche and R. Toder, 1998a Gene dosage in the evolution and function of mammalian sex chromosomes. Cytogenet Cell Genet **80:** 94-103.

Graves, J. A., M. J. Wakefield and R. Toder, 1998b The origin and evolution of the pseudoautosomal regions of human sex chromosomes. Hum Mol Genet **7:** 1991-1996.

Gray, I. C., D. A. Campbell and N. K. Spurr, 2000 Single nucleotide polymorphisms as tools in human genetics. Hum Mol Genet **9:** 2403-2408.

Gromoll, J., G. F. Weinbauer, H. Skaletsky, S. Schlatt, M. Rocchietti-March *et al.*, 1999 The Old World monkey DAZ (Deleted in AZoospermia) gene yields insights into the evolution of the DAZ gene cluster on the human Y chromosome. Hum Mol Genet **8:** 2017-2024.

Hanotte, O., C. L. Tawah, D. G. Bradley, M. Okomo, Y. Verjee *et al.*, 2000 Geographic distribution and frequency of a taurine Bos taurus and an indicine Bos indicus Y specific allele amongst sub-saharan African cattle breeds. Mol Ecol **9:** 387-396.

Hassanin, A., and E. J. Douzery, 1999 The tribal radiation of the family Bovidae (Artiodactyla) and the evolution of the mitochondrial cytochrome b gene. Mol Phylogenet Evol **13:** 227-243.

Hedges, S. B., 2000 Human evolution. A start for population genomics. Nature **408:** 652-653.

Hellborg, L., and H. Ellegren, 2003 Y chromosome conserved anchored tagged sequences (YCATS) for the analysis of mammalian male-specific DNA. Mol Ecol **12:** 283-291.

Hurles, M. E., and M. A. Jobling, 2001 Haploid chromosomes in molecular ecology: lessons from the human Y. Mol Ecol **10:** 1599-1613.

Hurst, L. D., 1998 Selfish genes and meiotic drive. Nature **391:** 223.

Hurst, L. D., 2001 Evolutionary genomics. Sex and the X. Nature **411:** 149-150.

Hurst, L. D., A. Atlan and B. O. Bengtsson, 1996 Genetic conflicts. Q Rev Biol **71:** 317-364.

Iannuzzi, L., G. P. Di Meo, A. Perucatti, A. Eggen, D. Incarnato *et al.*, 2001a A pericentric inversion in the cattle Y chromosome. Cytogenet Cell Genet **94:** 202-205.

Iannuzzi, L., L. Molteni, G. P. Di Meo, A. De Giovanni, A. Perucatti *et al.*, 2001b A case of azoospermia in a bull carrying a Y-autosome reciprocal translocation. Cytogenet Cell Genet **95:** 225-227.

Ingman, M., H. Kaessmann, S. Paabo and U. Gyllensten, 2000 Mitochondrial genome variation and the origin of modern humans. Nature **408:** 708-713.

Jakubiczka, S., F. Schnieders and J. Schmidtke, 1993 A bovine homologue of the human TSPY gene [published erratum appears in Genomics 1994 Jan 1;19(1):198]. Genomics **17:** 732-735.

Janecek, L. L., R. L. Honeycutt, R. M. Adkins and S. K. Davis, 1996 Mitochondrial gene sequences and the molecular systematics of the artiodactyl subfamily bovinae. Mol Phylogenet Evol **6:** 107-119.

Jobling, M. A., N. Bouzekri and P. G. Taylor, 1998a Hypervariable digital DNA codes for human paternal lineages: MVR-PCR at the Y-specific minisatellite, MSY1 (DYF155S1). Hum Mol Genet **7:** 643-653.

Jobling, M. A., and C. Tyler-Smith, 2000 New uses for new haplotypes the human Y chromosome, disease and selection. Trends Genet **16:** 356-362.

Jobling, M. A., G. A. Williams, G. A. Schiebel, G. A. Pandya, G. A. McElreavey *et al.*, 1998b A selective difference between human Y-chromosomal DNA haplotypes. Curr Biol **8:** 1391-1394.

Kadwell, M., M. Fernandez, H. F. Stanley, R. Baldi, J. C. Wheeler *et al.*, 2001 Genetic analysis reveals the wild ancestors of the llama and the alpaca. Proc R Soc Lond B Biol Sci **268:** 2575-2584.

Ke, Y., B. Su, X. Song, D. Lu, L. Chen *et al.*, 2001 African origin of modern humans in East Asia: a tale of 12,000 Y chromosomes. Science **292:** 1151-1153.

Kikkawa, Y., T. Takada, Sutopo, K. Nomura, T. Namikawa *et al.*, 2003 Phylogenies using mtDNA and SRY provide evidence for male-mediated introgression in Asian domestic cattle. Anim Genet **34:** 96-101.

Krings, M., A. Stone, R. W. Schmitz, H. Krainitzki, M. Stoneking *et al.*, 1997 Neandertal DNA sequences and the origin of modern humans. Cell **90:** 19-30.

Kuroda-Kawaguchi, T., H. Skaletsky, L. G. Brown, P. J. Minx, H. S. Cordum *et al.*, 2001 The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. Nat Genet **29:** 279-286.

Lahn, B. T., and D. C. Page, 1997 Functional coherence of the human Y chromosome. Science **278:** 675-680.

Lahn, B. T., and D. C. Page, 1999a Four evolutionary strata on the human X chromosome. Science **286:** 964-967.

Lahn, B. T., and D. C. Page, 1999b Retroposition of autosomal mRNA yielded testis-specific gene family on human Y chromosome [published erratum appears in Nat Genet 1999 Jun;22(2):209]. Nat Genet **21:** 429-433.

Lahn, B. T., N. M. Pearson and K. Jegalian, 2001 The human Y chromosome, in the light of evolution. Nat Rev Genet **2:** 207-216.

Lander, E. S., L. M. Linton, B. Birren, C. Nusbaum, M. C. Zody *et al.*, 2001 Initial sequencing and analysis of the human genome. Nature **409:** 860-921.

Lau, Y. F., 1999 Gonadoblastoma, testicular and prostate cancers, and the TSPY gene. Am J Hum Genet **64:** 921-927.

Lau, Y. F., P. M. Chou, J. C. Iezzoni, J. A. Alonzo and L. G. Komuves, 2000 Expression of a candidate gene for the gonadoblastoma locus in gonadoblastoma and testicular seminoma. Cytogenet Cell Genet **91:** 160-164.

Lau, Y. F., and J. Zhang, 2000 Expression analysis of thirty one Y chromosome genes in human prostate cancer. Mol Carcinog **27:** 308-321.

Lenstra, J. A., J. A. van Boxtel, K. A. Zwaagstra and M. Schwerin, 1993 Short interspersed nuclear element (SINE) sequences of the Bovidae. Anim Genet **24:** 33-39.

Liu, W. S., P. Mariani, C. W. Beattie, L. J. Alexander and F. A. Ponce De Leon, 2002 A radiation hybrid map for the bovine Y Chromosome. Mamm Genome **13:** 320-326.

Mackay, S., 2000 Gonadal development in mammals at the cellular and molecular levels. Int Rev Cytol **200:** 47-99.

Malaspina, P., F. Cruciani, B. M. Ciminelli, L. Terrenato, P. Santolamazza *et al.*, 1998 Network analyses of Y-chromosomal types in Europe, northern Africa, and western Asia reveal specific patterns of geographic distribution. Am J Hum Genet **63:** 847-860.

Manz, E., F. Schnieders, A. M. Brechlin and J. Schmidtke, 1993 TSPY-related sequences represent a microheterogeneous gene family
organized as constitutive elements in DYZ5 tandem repeat units on the human Y chromosome. Genomics **3:** 726-731.

Margarit, E., A. Guillen, C. Rebordosa, J. Vidal-Taboada, M. Sanchez *et al.*, 1998 Identification of conserved potentially regulatory sequences of the SRY gene from 10 different species of mammals. Biochem Biophys Res Commun **245:** 370-377.

Marshall Graves, J. A., 2000 Human Y chromosome, sex determination, and spermatogenesis- a feminist view. Biol Reprod **63:** 667-676.

Matthee, C. A., J. D. Burzlaff, J. F. Taylor and S. K. Davis, 2001 Mining the mammalian genome for artiodactyl systematics. Syst Biol **50:** 367-390.

Matthee, C. A., and S. K. Davis, 2001 Molecular insights into the evolution of the family Bovidae: a nuclear DNA perspective. Mol Biol Evol **18:** 1220-1230.

Matthews, M. E., and K. C. Reed, 1992 Sequences from a family of bovine Y-chromosomal repeats. Genomics **13:** 1267-1273.

Miller, J. R., and M. Koopman, 1990 Isolation and characterization of two male-specific DNA fragments from the bovine gene. Anim Genet **21:** 77-82.

Mitchell, M. J., S. A. Wilcox, J. M. Watson, J. L. Lerner, D. R. Woods *et al.*, 1998 The origin and loss of the ubiquitin activating enzyme gene on the mammalian Y chromosome. Hum Mol Genet **7:** 429-434.

Morrish, B. C., and A. H. Sinclair, 2002 Vertebrate sex determination: many means to an end. Reproduction **124:** 447-457.

Nijman, I. J., D. G. Bradley, O. Hanotte, M. Otsen and J. A. Lenstra, 1999 Satellite DNA polymorphisms and AFLP correlate with Bos indicus-taurus hybridization. Anim Genet **30:** 265-273.

Nijman, I. J., M. Otsen, E. L. C. Verkaar, C. de Ruijter, E. Hanekamp *et al.*, 2002a Hybridization of banteng (Bos javanicus) and zebu (Bos indicus) revealed by mitochondrial DNA, satellite DNA, AFLP and microsatellites. in press.

Nijman, I. J., P. van Tessel and J. A. Lenstra, 2002b SINE Retrotransposition During the Evolution of the Pecoran Ruminants. J Mol Evol **54:** 9-16.

Nikaido, M., A. P. Rooney and N. Okada, 1999 Phylogenetic relationships among cetartiodactyls based on insertions of short and long interpersed elements: hippopotamuses are the closest extant relatives of whales. Proc Natl Acad Sci U S A **96:** 10261-10266.

Oates, R. D., S. Silber, L. G. Brown and D. C. Page, 2002 Clinical characterization of 42 oligospermic or azoospermic men with microdeletion of the AZFc region of the Y chromosome, and of 18 children conceived via ICSI. Hum Reprod **17:** 2813-2824.

O'Neill, R. J., M. D. Eldridge and J. A. Graves, 2001 Chromosome heterozygosity and de novo chromosome rearrangements in mammalian interspecies hybrids. Mamm Genome **12:** 256-259.

Oota, H., W. Settheetham-Ishida, D. Tiwawech, T. Ishida and M. Stoneking, 2001 Human mtDNA and Y-chromosome variation is correlated with matrilocal versus patrilocal residence. Nat Genet **29:** 20-21.

Partridge, L., and L. D. Hurst, 1998 Sex and conflict. Science **281:** 2003-2008.

Pecon Slattery, J., and S. J. O'Brien, 1998 Patterns of Y and X chromosome DNA sequence divergence during the Felidae radiation. Genetics **148:** 1245-1255.

Perret, J., Y. C. Shia, R. Fries, G. Vassart and M. Georges, 1990 A polymorphic satellite sequence maps to the pericentric region of the bovine Y chromosome. Genomics **6:** 482-490.

Polziehn, R. O., C. Strobeck, J. Sheraton and R. Beech, 1995 Bovine mtDNA discovered in North American bison populations. Conservation Biology**:** 1638-1643.

Pomiankowski, A., and L. D. Hurst, 1993 Evolutionary genetics. Siberian mice upset Mendel. Nature **363:** 396-397.

Popescu, C. P., C. Cotinot, J. Boscher and M. Kirszenbaum, 1988 Chromosomal localization of a bovine male specific probe. Ann Genet **31:** 39-42.

Queipo, G., C. Zenteno, R. Pena, K. Nieto, A. Radillo *et al.*, 2002 Molecular analysis in true hermaphroditism: demonstration of low-level hidden mosaicism for Y-derived sequences in 46,XX cases. Hum Genet **111:** 278-283.

Quilter, C. R., S. C. Blott, A. J. Mileham, N. A. Affara, C. A. Sargent *et al.*, 2002 A mapping and evolutionary study of porcine sex chromosome genes. Mamm Genome **13:** 588-594.

Quintana-Murci, L., C. Krausz and K. McElreavey, 2001 The human Y chromosome: function, evolution and disease. Forensic Sci Int **118:** 169-181.

Rice, W. R., 1987 The accumulation of sexually antagonistic genes as a selective agent promoting the evolution of reduced recombination between primitive sex-chromosomes. Evolution **41:** 911-914.

Rice, W. R., and B. Holland, 1997 The enemies within: intergenomic conflict, interlocus contest evolution (ICE), and the intraspecific Red Queen. Behav Ecol Sociobiol**:** 1-10.

Ridley, M., 1994 The Red Queen. Penguin books.

Ridley, M., 1996 Evolution 2nd ed. Blackwell science, Cambridge, USA.

Ritz, L. R., M. L. Glowatzki-Mullis, D. E. MacHugh and C. Gaillard, 2000 Phylogenetic analysis of the tribe Bovini using microsatellites. Anim Genet **31:** 178-185.

Romualdi, C., D. Balding, I. S. Nasidze, G. Risch, M. Robichaux *et al.*, 2002 Patterns of human diversity, within and among continents, inferred from biallelic DNA polymorphisms. Genome Res **12:** 602-612.

Rozen, S., H. Skaletsky, J. D. Marszalek, P. J. Minx, H. S. Cordum *et al.*, 2003 Abundant gene conversion between arms of palindromes in human and ape Y chromosomes. Nature **423:** 873-876.

Schubert, S., F. Dechend, B. Skawran, B. Kunze, H. Winking *et al.*, 2000 Silencing of the Y-chromosomal gene tspy during murine evolution. Mamm Genome **11:** 288-291.

Schwerin, M., D. S. Gallagher, Jr., J. R. Miller and P. D. Thomsen, 1992 Mapping of repetitive bovine DNA sequences on cattle Y chromosomes. Cytogenet Cell Genet **61:** 189-194.

Seielstad, M. T., E. Minch and L. L. Cavalli-Sforza, 1998 Genetic evidence for a higher female migration rate in humans. Nat Genet **20:** 278-280.

Skaletsky, H., T. Kuroda-Kawaguchi, P. J. Minx, H. S. Cordum, L. Hillier *et al.*, 2003 The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature **423:** 825-837.

Sonstegard, T. S., W. Barendse, G. L. Bennett, G. A. Brockmann, S. Davis *et al.*, 2001 Consensus and comprehensive linkage maps of the bovine sex chromosomes. Anim Genet **32:** 115-117.

Templeton, A., 2002 Out of Africa again and again. Nature **416:** 45-51.

Thomsen, P. D., and C. B. Jorgensen, 1994 Distribution of two conserved, male-enriched repeat families on the Bos taurus Y chromosome. Mamm Genome **5:** 171-173.

Tilford, C. A., T. Kuroda-Kawaguchi, H. Skaletsky, S. Rozen, L. G. Brown *et al.*, 2001a A physical map of the human Y chromosome. Nature **409:** 943-945.

Tilford, C. A., T. Kuroda-Kawaguchi, H. Skaletsky, S. Rozen, L. G. Brown *et al.*, 2001b A physical map of the human Y chromosome. Nature **409:** 943-945.

Toder, R., M. J. Wakefield and J. A. Graves, 2000 The minimal mammalian Y chromosome - the marsupial Y as a model system. Cytogenet Cell Genet **91:** 285-292.

Tosi, A. J., J. C. Morales and D. J. Melnick, 2000 Comparison of Y Chromosome and mtDNA Phylogenies Leads to Unique Inferences of Macaque Evolutionary History. Mol Phylogenet Evol **17:** 133-144.

Troy, C. S., D. E. MacHugh, J. F. Bailey, D. A. Magee, R. T. Loftus *et al.*, 2001 Genetic evidence for Near-Eastern origins of European cattle. Nature **410:** 1088-1091.

Van Hooft, P., 2001 Population genetics of the African buffalo. Thesis Wageningen University, Department of Environmental Sciences, Tropical Nature conservationand vertebrate ecology group**:** 61-83.

Van Hooft, W. F., A. F. Groen and H. H. Prins, 2002 Phylogeography of the African buffalo based on mitochondrial and Y-chromosomal loci: Pleistocene origin and population expansion of the Cape buffalo subspecies. Mol Ecol **11:** 267-279.

Veitia, R. A., L. Salas-Cortes, C. Ottolenghi, E. Pailhoux, C. Cotinot *et al.*, 2001 Testis determination in mammals: more questions than answers. Mol Cell Endocrinol **179:** 3-16.

Verkaar, E. L. C., I. J. Nijman, M. Beeke, E. Hanekamp and J. A. Lenstra, 2002a Maternal and paternal lineages in cross-breeding bovine species: The unusual phylogeny of wisent. submitted.

Verkaar, E. L. C., I. J. Nijman, K. Boutaga and J. A. Lenstra, 2002b Differentiation of cattle species in beef by PCR-RFLP of mitochondrial and satellite DNA. Meat Science **60:** 365-369.

Vogel, T., H. Boettger-Tong, I. Nanda, F. Dechend, A. I. Agulnik *et al.*, 1998a A murine TSPY. Chromosome Res **1:** 35-40.

Vogel, T., S. Borgmann, F. Dechend, W. Hecht and J. Schmidtke, 1997a Conserved Y-chromosomal location of TSPY in Bovidae. Chromosome Res **5:** 182-185.

Vogel, T., F. Dechend, E. Manz, C. Jung, S. Jakubiczka *et al.*, 1997b Organization and expression of bovine TSPY. Mamm Genome **8:** 491-496.

Vogel, T., O. Dittrich, Y. Mehraein, F. Dechend, F. Schnieders *et al.*, 1998b Murine and human TSPYL genes: novel members of the TSPY-SET-NAP1L1 family. Cytogenet Cell Genet **81:** 265-270.

Vogel, T., and J. Schmidtke, 1998 Structure and function of TSPY, the Y-chromosome gene coding for the "testis-specific protein". Cytogenet Cell Genet **80:** 209-213.

Wang, P. J., J. R. McCarrey, F. Yang and D. C. Page, 2001 An abundance of X-linked genes expressed in spermatogonia. Nat Genet **27:** 422-426.

Wang, X., J. Zhang and Y. P. Zhang Yp, 2002 Erratic Evolution of SRY in Higher Primates. Mol Biol Evol **19:** 582-584.

Ward, T. J., J. P. Bielawski, S. K. Davis, J. W. Templeton and J. N. Derr, 1999 Identifiction of domestic cattle hybrids in wild cattle and bison species: a general approach using mtDNA markers and the parametric bootstrap. Animal Conservation**:** 51-57.

Ward, T. J., L. C. Skow, D. S. Gallagher, R. D. Schnabel, C. A. Nall *et al.*, 2001 Differential introgression of uniparentally inherited markers in bison populations with hybrid ancestries. Anim Genet **32:** 89-91.

Waters, P. D., B. Duffy, C. J. Frost, M. L. Delbridge and J. A. Graves, 2001 The human Y chromosome derives largely from a single autosomal region added to the sex chromosomes 80-130 million years ago. Cytogenet Cell Genet **92:** 74-79.

Wyckoff, G. J., J. Li and C. I. Wu, 2002 Molecular evolution of functional genes on the Mammalian y chromosome. Mol Biol Evol **19:** 1633-1636.

Xiao, C., K. Tsuchiya and S. Sutou, 1998 Cloning and mapping of bovine ZFX gene to the long arm of the X-chromosome (Xq34) and homologous mapping of ZFY gene to the distal region of the short arm of the bovine (Yp13), ovine (Yp12-p13), and caprine (Yp12-p13) Y chromosome. Mamm Genome **9:** 125-130.

Yu, Y., L. Nie, Z. Q. He, J. K. Wen, C. S. Jian *et al.*, 1999 Mitochondrial DNA variation in cattle of south China: origin and introgression. Anim Genet **30:** 245-250.

CHAPTER 2A

Differentiation of cattle species in beef by PCR-RFLP of mitochondrial and satellite DNA

E.L.C.Verkaar, I.J.Nijman, K.Boutaga and J.A.Lenstra (2002), *Meat Science* **60**, 365-369

Abstract

Methods currently used for the identification of the species origin of meat or tissue samples have not been validated for other bovine species than taurine cattle or water buffalo. These methods also do not discriminate between the different bovine species that are used as source of beef. Here we describe two complementary methods for detection and differentiation of bovine species, which are based on mutations in mitochondrial DNA and centromeric satellite DNA, respectively. The analysis of satellite DNA is especially relevant for the identification of animals that are of hybrid origin.

1. Introduction

Identification of the species origin in meat samples is relevant for economical, religious or public health concerning reasons (Buntjer *et al.* 1995; Lenstra and Bradley 1999). Species detection is also relevant for wildlife management, which so far has not received the level of attention as food inspection or human forensic science (Dove 1999).

Modern methods for meat identification are based on DNA analysis. Hybridization of DNA extracted from meat to probes recognising the species-specific satellite repeats can be used to discriminate related species e.g. sheep versus cattle; chicken versus turkey (Buntjer *et al.* 1998; Hunt *et al.* 1997). Alternatively, PCR analysis of mitochondrial DNA (Chikuni *et al.* 1994; Dickinson *et al.* 1995; Guglich *et al.* 1994; Matsunaga *et al.* 1999; Partis *et al.* 2000; Wolf *et al.* 1999) is used. In most cases both methods are adequate to identify the species origin of meat in processed meat samples.

However, most methods are only partially suitable for the analysis of beef samples. First, the most commonly used PCR-primers (Kocher *et al.* 1989; Meyer *et al.* 1995) are based on the human mitochondrial cytochrome *b* sequence and have several mismatches relative to the bovine sequence. As a consequence, these primers are not suitable for analysis of mixed species origin or samples in which most DNA is degraded. Tartaglia (Tartaglia *et al.* 1998) designed a dedicated assay for a sensitive but exclusive detection of bovine material. Secondly, the PCR tests (Matsunaga *et al.* 1999; Meyer *et al.* 1995) have been val-

idated only for the two most common bovine species, taurine cattle and water buffalo. Meat from other bovine species like zebu, banteng, gaur, bison or yak is consumed in several parts of the world, either as a substitute of taurine beef or as a high quality beef variant. Thirdly, no method is available to discriminate between meat from other bovine species. PCR tests for differentiation of mitochondrial DNA from cattle and bison have been described for conservation purposes (Murray *et al.* 1995; Ward *et al.* 1999).

Finally, hybridization of different bovine species is not uncommon and invalidates any test based on the maternally transmitted mitochondrial DNA. Several African zebu breeds are taurine-indicine hybrids (Nijman *et al.* 1999) while taurine-yak, banteng-zebu as well as gaur-zebu hybridization occurs in various regions in Asia (Bradley *et al.* 1994; Lenstra and Bradley 1999). Cattle introgression has occurred in the American bison population (Polziehn *et al.* 1995; Ward *et al.* 1999). The American Beefalo cattle is bred for its beef quality and resulted from crossings of bison with cattle. Here we describe two complementary methods for bovine species identification. The first method is based on species-specific mutations in the mitochondrial cytochrome *b* and cytochrome oxidase II genes, extending the range of applications of the mitochondrial PCR-RFLP assay. Secondly, as confirmatory method, we describe SFLP (satellite fragment length polymorphism), a PCR-RFLP on centromeric satellite DNA (Nijman *et al.* 1999; Nijman and Lenstra 2001), which offers the additional advantage of detecting interspecies hybridization.

2. Material and Methods

2.1. Samples and DNA isolation

Blood or tissue (muscle or liver) from bovine species was collected from taurine cattle (*Bos taurus*), zebu (*Bos indicus*), banteng (*Bos javanicus*), gaur (*Bos frontalis*), gayal (*Bos gaurus*), yak (*Bos grunniens*), bison (*Bison bison*), wisent (*Bison bonasus*), water buffalo (*Bubalus bubalis*) and African buffalo (*Syncerus caffer*). DNA from blood was isolated using the guanidium-isothiocyanate protocol as described (Ciulla *et al.* 1988). DNA from tissue was isolated by proteinase-K/SDS extraction (Sambrook *et al.* 1989).

Table 1. Oligonucleotides used as PCR primers.

Target	Primer sequence 5'-3' (left and right primer, respectively)	Amplicon length (bp)
cytochrome *b*	ACAAATCCTCACAGGCCTATTC TAGGACGTATCCTATGAATGCT	271
cytochrome oxidase II	ATGGCATATCCCATACAACTAG ACTTTAGTGGGACTAACTCAAG	651
satellite IV	AAGCTTGTGACAGATAGAACGAT CAAGCTGTCTAGAATTCAGGGA	604
satellite 1.711b	CTGGGTGTGACAGTGTTTAAC TGATCCAGGGTATTCGAAGGA	822

Table 2. Genbank entries of mitochondrial sequences. Asterisks denote new data generated in order to resolve discrepancies between predicted and observed restriction digests.

Species	**cytochrome *b***	**Cytochrome oxidase II**
taurine cattle	D34635	M10544
zebu	AF348597	AF348595
banteng	D34636	U18821
gaur	AF348593	AF348592
gayal	AF348596	U18818
yak	Y16063	AF348594
bison	Y16060	U62568
wisent	Y16061	U62567
water buffalo	X78960	U18822
african buffalo	Y16056	U18825

2.2. PCR-RFLP

PCR was performed in a total volume of 25 µl, containing 50 ng genomic DNA and 50 ng of both primers in Taq DNA polymerase buffer (Promega, Madison, USA) with 1.5 mM $MgCl_2$, 0.2 mM dNTP's (Promega) and 1.25 U Taq DNA polymerase. The following program was used: predenaturation for 2 min at 95^{o}C, followed 25 PCR cycles of 15 s 92^{o}C, 30 s at 58^{o}C and 45 s at 72^{o}C and by a final extension of 5 min at 72^{o}C. For restriction endonuclease analysis with *Ava*III, *Bam*HI, *Eco*RI, *Hin*dIII, *Hin*fI, *Stu*I, *Taq*I, *Xba*I (Amersham-Pharmacia, Amersham, UK; Uppsala, Sweden), *Ban*II or its isoschizomer *Tru*9 (Fermentas, Vilnius, Lithuania), *Mse*I (New England BioLabs, Beverley, USA) 5 µl of the PCR product was digested by addition of 5-10 U of restriction endonuclease and the recommended concentrated reaction buffer. Samples were digested for 3 h at 37^{o}C, 60^{o}C (*Taq*I) or 65^{o}C (*Tru9*) and fractionated on a 2% agarose (SphaeroQ, Leiden, The Netherlands) / 0.5´TBE gel. For sequencing, PCR products were separated on a 2% agarose / 0.5´TBE gel, excised and purified with the QIAquick system (QIAGEN Inc. Valencia, CA, USA) following the manufacturers protocol. Sequencing was performed using the Cy5 Big Dye terminator kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) on an ABI Prism 310 sequencer (Perkin-Elmer).

3. Results and discussion

3.1. Mitochondrial DNA

Sequences of cytochrome *b* (Hassanin and Douzery 1999; Schreiber *et al.* 1999) and cytochrome oxidase II (Janecek *et al.* 1996) from bovine species were retrieved from Genbank (Table 2) and aligned (Corpet 1988). For a few species the sequences were partially re-analysed to resolve discrepancies. The commonly used downstream cytochrome *b* primer (Meyer *et al.* 1995) has 4 mismatches with the bovine sequence, one of which is 3 nt from the 3' end. Therefore, new cytochrome *b* primers for the bovine species were designed (Table 1). These primers amplified the cytochrome *b* fragment from all artiodactyls tested (bovines, sheep, goat, nilgai, rein deer and pig) and from horse. A faint product was obtained with human DNA and chicken DNA was negative (results not shown). The

cytochrome oxidase II primers (Janecek *et al.* 1996) have a narrower species range and generated a product only with DNA from bovine species or with DNA from the nilgai (*Boselaphus tragocamelus*), also belonging to the Bovinae subtribe.

Meyer and co-authors identified two diagnostic restriction sites present in the cytochrome *b* genes from taurine cattle and water buffalo, *Hae*III and *Alu*I. The *Hae*III site is present in the sequence of all bovine species except the African buffalo, but this site is not within the cytochrome *b* amplicon used in our assay. The *Alu*I site is conserved in all bovine species (Table 3). As reported previously (Meyer *et al.* 1995), a *Hin*fI site discriminates cattle from water buffalo. However, this site is lacking in other bovine species that are also used as source of beef. For a complete discrimination of bovine species, other species-specific sites were identified in the alignments (Table 3). All cleavage patterns predicted from the sequence were checked experimentally.

By combining diagnostic restriction sites of two mitochondrial genes, the mitochondrial PCR-RFLP assay gives a positive identification of all bovine species via the presence of a restriction site, except that taurine cattle and zebu are not differentiated. In all cases except gayal versus banteng and gayal versus wisent the species identification can be based on two mutations.

Recently, a PCR-RFLP test on the mitochondrial 12S RNA was described that differentiated taurine cattle and Asian zebu (Meirelles *et al.* 2001). However, African zebu breeds emerged by introgression and have retained mitochondrial DNA of the taurine type.

3.2. Satellite DNA

A drawback inherent to any mitochondrial assay is that in cases of species hybridization only the maternal lineage is identified. Therefore, a confirmatory assay is needed that is based on nuclear DNA. Satellite DNA consists of centromeric tandem repeats and occupies up to 20% of the cattle genome (Jobse *et al.* 1995; Nijman and Lenstra 2001; Skowronski *et al.* 1984). As a result of concerted evolution, the sequences become species-specific (Elder and Turner 1995). The bovine species share similar satellite families, but the frequencies of sequence variants is variable, which can be detected by satellite fragment length polymorphism assays (Table 4: SFLP; Nijman *et al.* 1999). As an example, Figure 1a shows a differentiation of bisons (three animals) and cattle (three animals from different

breeds) by SFLP. A sequence variant of satellite IV with an *Mse*I site is clearly more frequent in bison than cattle, while a *Taq*I variant of the same satellite predominates in cattle. So far, we never observed intraspecies variation of SFLP patterns (Nijman *et al.* 1999). Both the bison and cattle satellites are also amplified in a mixture of DNA from both species (Figure 1b).

Figure 2 shows that SFLP patterns of a commercially obtained bison beef sample are identical to the patterns of purified bison DNA and confirm the mitochondrial *Stu*I PCR-RFLP. However, about 6% of the American bison populations carries mitochondria of taurine origin (Ward *et al.* 1999). For these animals a mitochondrial assay will be misleading, while satellite-based assays would still indicate the predominantly bison origin of the nuclear genome.

Another case of bovine species hybridization is shown in Figure 3. Bali cattle is considered to be domesticated banteng, which is apparent from the satellite IV *Mse*I pattern. However, the Hin*fI* and *Taq*I digests of cytochrome *b* amplicons clearly show zebu-specific patterns, indicating that this Malaysian Bali cattle individual is a hybrid of banteng and zebu. Nijman *et al.*(1999) has used a *Sau*3AI-1711b SFLP assay to analyse the various

Table 3. Lengths of fragments generated by digestion of mitochondrial PCR products with the indicated restriction enzyme.

Species	**cytochrome *b***						**cytochrome oxidase II**
	***Alu*I**	***Xba*I**	***Stu*I**	***Bam*HI**	***Hin*fI**	***Taq*I**	***Eco*RI**
taurine cattle	131, 140	198,73	271	271	101, 170	271	244, 407
zebu	131, 140	198, 73	271	271	101, 170	271	244, 407
banteng	131, 140	271	171, 100	271	271	108, 163	651
gaur, gayal	131, 140	271	171, 100	271	271	271	651
yak	131, 140	271	271	271	271	271	244, 407
bison	131, 140	271	171, 100	271	101, 170	271	244, 407
wisent	131, 140	198, 73	171, 100	271	271	271	651
water buffalo	131, 140	198, 73	271	271	271	108, 163	244, 407
african buffalo	131, 140	271	171, 100	184, 87	271	108, 163	244, 407

Table 4. Lengths of fragments generated by digestion of satellite DNA PCR products with the indicated restriction enzymes. Fragments that are underlined are relatively intense.

species	satellite IV		satellite 1.711b		
	BanII	***MseI***	***Hin*dIII**	***Taq*I**	***Sau* 3AI**
taurine cattle	604, 435, 169	604	822, 500, 322	809, 552, 257	822
zebu	604, 435, 169	604	822, 500, 322	809, 552, 257	822, 741, 81
banteng	604, 435, 169	604, 472, 132	822, 500, 322	822, 553, 269	822
gaur, gayal	604, 435, 169	604, 472, 132	822, 500, 322	822, 553, 269	822
yak	604, 435, 169	604, 529, 467, 137, 75, 63	822	822	822
bison	604, 435, 169	604, 529, 467, 137, 75, 63	822, 500, 322	822	822
wisent	604, 435, 169	604, 529, 467, 137, 75, 63	822, 500, 322	822	822

degrees of taurine-indicine (zebu) hybridization in African cattle breeds.

The SFLP test relies on the simultaneous amplification of several, slightly different repeated elements and is based on semi-quantitative rather than absolute differences in the occurrence of restriction sites. Consequently, it has not been designed for the detection of admixtures or analysis of degraded DNA and is most useful for unprocessed samples that originate from a single animal.

We note that also nuclear transplantation from the endangered gaur species in taurine oocytes (Lanza *et al.* 2000) results in animals for which the species origin can only be verified by an analysis of nuclear DNA like the SFLP.

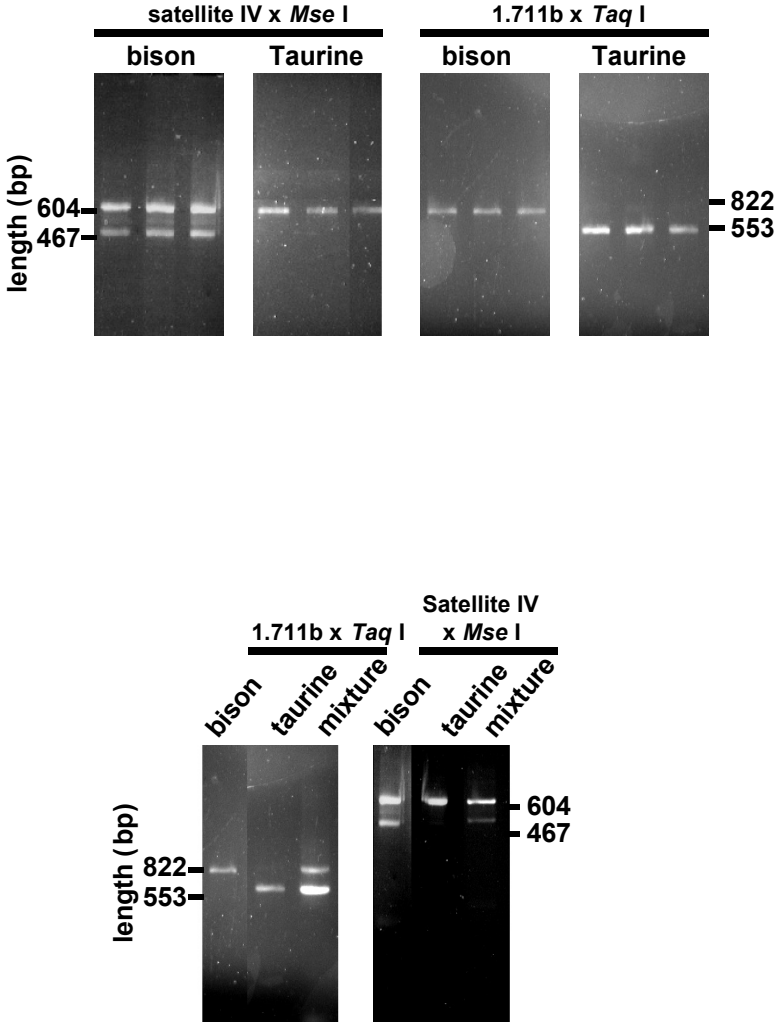

Figure 1(a). SFLP patterns of bison and different breeds of taurine cattle (from left to right; Holstein-Friesian, Meuse-Rhine-Yssel and Jersey, respectively). **(b).** Analysis of 1:1 mixtures of bison and taurine samples. Fragments of satellite DNA were amplified and cleaved with the indicated restriction enzymes.

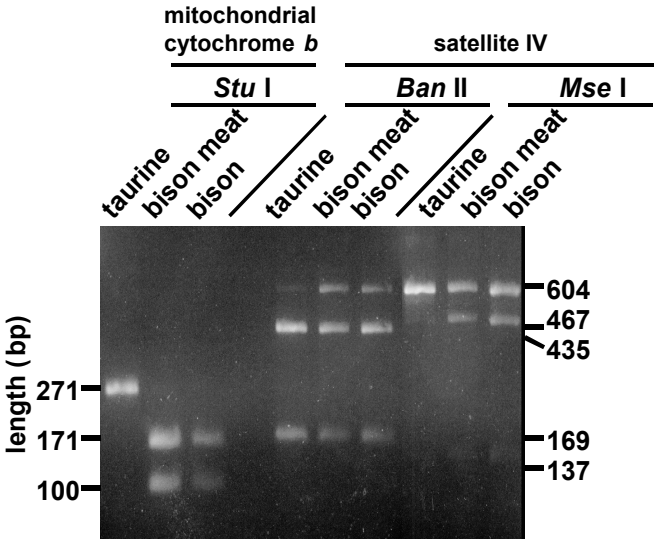

Figure 2. Mitochondrial PCR-RFLP and SFLP patterns with the indicated restriction sites of commercially obtained bison meat sample compared with the ox and bison reference samples.

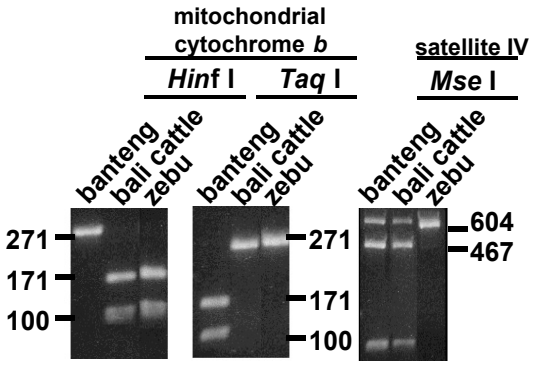

Figure 3. Mitochondrial PCR-RFLP and SFLP with the individual restriction sites of a Bali cattle individual compared with banteng and zebu reference samples.

4. Conclusion

All bovine species can be identified by convenient, sensitive and versatile PCR-RFLP assays. This may serve as follow-up if an *Alu*I digestion of the mitochondrial cytochrome *b* (Meyer *et al.* 1995) indicates the presence of bovine material, without differentiating between bovine species. We propose the following recommendations in order to ensure validity of the assays described in this paper. Firstly, all relevant reference animals should be tested in parallel. Secondly, the differentiation should be based on at least two different restriction enzyme sites in order to exclude intraspecies polymorphism. Thirdly, at least one SFLP assay should be used if species hybridization has to be excluded. These considerations may be of general relevance for the discrimination of other closely related species.

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References

Bradley, D. G., D. E. MacHugh, R. T. Loftus, R. S. Sow, C. H. Hoste *et al.*, 1994 Zebu-taurine variation in Y chromosomal DNA: a sensitive assay for genetic introgression in west African trypanotolerant cattle populations. Anim Genet **25:** 7-12.

Buntjer, J. B., J. A. Lenstra and N. Haagsma, 1995 Rapid species identification in meat by using satellite DNA probes. Z Lebensm Unters Forsch **201:** 577-582.

Buntjer, J. B., I. J. Nijman, C. Zijlstra and J. A. Lenstra, 1998 A satellite DNA element specific for roe deer (Capreolus capreolus). Chromosoma **107:** 1-5.

Chikuni, K., T. Tabata, M. Kosugiyama, M. Momma and M. Saito, 1994 Polymerase chain reaction assay for detection of sheep and goat meats. Meat Sci**:** 337-345.

Ciulla, T. A., R. M. Sklar and S. L. Hauser, 1988 A simple method for DNA purification from peripheral blood. Anal Biochem **174:** 485-488.

Corpet, F., 1988 Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res **16:** 10881-10890.

Dickinson, J. H., R. G. Kroll and K. A. Grant, 1995 The direct application of the polymerase chain reaction to DNA extracted from foods. Lett Appl Microbiol **20:** 212-216.

Dove, A., 1999 The long arm of DNA. Nat Biotechnol **17:** 649-651.

Elder, J. F., Jr., and B. J. Turner, 1995 Concerted evolution of repetitive DNA sequences in eukaryotes. Q Rev Biol **70:** 297-320.

Guglich, E. A., P. J. Wilson and B. N. White, 1994 Forensic application of repetitive DNA markers to the species identification of animal tissues. J Forensic Sci **39:** 353-361.

Hassanin, A., and E. J. Douzery, 1999 The tribal radiation of the family Bovidae (Artiodactyla) and the evolution of the mitochondrial cytochrome b gene. Mol Phylogenet Evol **13:** 227-243.

Hunt, D., H. C. Parkes and I. D. Davies, 1997 Identification of the species origin of raw and cooked meat products using oligonucleotide probes. J Food Chem**:** 437-422.

Janecek, L. L., R. L. Honeycutt, R. M. Adkins and S. K. Davis, 1996 Mitochondrial gene sequences and the molecular systematics of the artiodactyl subfamily bovinae. Mol Phylogenet Evol **6:** 107-119.

Jobse, C., J. B. Buntjer, N. Haagsma, H. J. Breukelman, J. J. Beintema *et al.*, 1995 Evolution and recombination of bovine DNA repeats. J Mol Evol **41:** 277-283.

Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Paabo *et al.*, 1989 Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci U S A **86:** 6196-6200.

Lanza, R. P., B. L. Dresser and P. Damiani, 2000 Cloning Noah's ark. Sci Am **283:** 84-89.

Lenstra, J. A., and D. G. Bradley, 1999 Systematics and phylogeny of cattle. CAB Intl. Ed. by R. Fries and A. Ruvenski**:** 1-14.

Matsunaga, T., K. Chikuni, R. Tanabe, S. Muroya, K. Shibata *et al.*, 1999 A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Science**:** 143-148.

Meirelles, F. V., V. Bordignon, Y. Watanabe, M. Watanabe, A. Dayan *et al.*, 2001 Complete Replacement of the Mitochondrial Genotype in a Bos indicus Calf Reconstructed by Nuclear Transfer to a Bos taurus Oocyte. Genetics **158:** 351-356.

Meyer, R., C. Hofelein, J. Luthy and U. Candrian, 1995 Polymerase chain reaction-restriction fragment length polymorphism analysis: a simple method for species identification in food. J AOAC Int **78:** 1542-1551.

Murray, B. W., R. A. McClymont and C. Strobeck, 1995 Forensic identification of ungulate species using restriction digests of PCR-amplified mitochondrial DNA. J Forensic Sci **40:** 943-951.

Nijman, I. J., D. G. Bradley, O. Hanotte, M. Otsen and J. A. Lenstra, 1999 Satellite DNA polymorphisms and AFLP correlate with Bos indicus-taurus hybridization. Anim Genet **30:** 265-273.

Nijman, I. J., and J. A. Lenstra, 2001 Mutation and recombination in cattle satellite DNA: a feedback model for the evolution of satellite DNA repeats. J Mol Evol **52:** 361-371.

Partis, L., D. Croan, Z. Guo, R. Clark, T. Coldham *et al.*, 2000 Evaluation of a DNA fingerprinting method for determining the species origin of meats. Meat Sci**:** 369-376.

Polziehn, R. O., C. Strobeck, J. Sheraton and R. Beech, 1995 Bovine mtDNA discovered in North American bison populations. Conservation Biology**:** 1638-1643.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 Molecular cloning. A laboratory manual. Cold spring harbor laboratory press, Cold spring harbor, USA.

Schreiber, A., I. Seibold, G. Notzold and M. Wink, 1999 Cytochrome b gene haplotypes characterize chromosomal lineages of anoa, the Sulawesi dwarf buffalo (Bovidae: Bubalus sp.). J Hered **90:** 165-176.

Skowronski, J., A. Plucienniczak, A. Bednarek and J. Jaworski, 1984 Bovine 1.709 satellite. Recombination hotspots and dispersed repeated sequences. J Mol Biol **177:** 399-416.

Tartaglia, M., E. Saulle, S. Pestalozza, L. Morelli, G. Antonucci *et al.*, 1998 Detection of bovine mitochondrial DNA in ruminant feeds: a molecular approach to test for the presence of bovine-derived materials. J Food Prot **61:** 513-518.

Ward, T. J., J. P. Bielawski, S. K. Davis, J. W. Templeton and J. N. Derr, 1999 Identifiction of domestic cattle hybrids in wild cattle and bison species: a general approach using mtDNA markers and the parametric bootstrap. Animal Conservation**:** 51-57.

Wolf, C., J. Rentsch and P. Hubner, 1999 PCR-RFLP analysis of mitochondrial DNA: a reliable method for species identification. J Agric Food Chem **47:** 1350-1355.

CHAPTER 2B

Paternally inherited markers in bovine hybrid populations

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Abstract

The genetic integrity of cross-fertile bovine or cattle-like species may be endangered by species-hybridisation. Previously, AFLP (amplified fragment length polymorphism), SFLP (satellite fragment length polymorphism) and microsatellite assays have been used to analyze the species composition of nuclear DNA in taurine cattle, zebu, banteng and bison populations, while mitochondrial DNA reveals the origin of the maternal lineages. Here we describe species-specific markers of the paternally transmitted Y-chromosome for the direct detection of male-mediated introgression. Convenient PCR-RFLP and competitive PCR assays are shown to differentiate the Y-chromosomes of taurine cattle, American bison and European bison and to detect the banteng origin of Indonesian Madura and Bali cattle bulls.

1. Introduction

Domestication of several bovine species has made the tribe of the *Bovini* one of the numerically most important mammalian taxa with 11 extant species: ox (*Bos taurus*), zebu (*Bos indicus*), gayal (*Bos frontalis*), gaur (*Bos gaurus*), banteng (*Bos javanicus*), bison (*Bison bison*), wisent (*Bison bonasus*) or European bison, yak (*Bos grunniens*), water buffalo (*Bubalus bubalis*), African buffalo (*Syncerus caffer*) and anoa (*Bubalus depressicornis*) (Lenstra and Bradley 1999). Speciation of the *Bos* and *Bison* species is incomplete and hybridisation of these species occurs world-wide (Bongso *et al.* 1988; Felius 1985; Lenstra and Bradley 1999; Nijman *et al.* 1999; Ward *et al.* 1999; Ward *et al.* 2001). Female progeny as well as the male taurine-zebu, gaur-gayal and bison-wisent hybrids are fertile, while fertility of other male hybrid offspring can be restored by repeated back crossings. Introgression in the wild species may compromise their genetic integrity. Conversely, organized crossing of wild bovines in domestic populations may create animals or even new breeds with unique properties. In Africa, introgression of Indian zebu bulls in taurine herds has occurred (Bradley *et al.* 1996). The Chinese yakow is a yak-ox hybrid, which is held at altitudes between the habitat ranges of the parent species (Felius 1985). The South-East Asian Selembu dairy and beef cattle result from gayal-zebu crossings (Felius 1985).

Taurine mitochondria have been found in wild North-American bison populations (Ward *et al.* 1999), while the beefalo may be regarded as a domestic hybrid breed of bisons and taurine cattle.

Several types of molecular markers have been used for the detection of bovine species hybridisation. Mitochondrial DNA is informative as marker of the maternal lineage (Bradley *et al.* 1996; Janecek *et al.* 1996; Verkaar *et al.* 2002; Ward *et al.* 1999). Amplified fragment length polymorphism (AFLP), satellite fragment length polymorphism's (SFLP) and species-specific autosomal microsatellite alleles may serve as autosomal markers (Buntjer *et al.* 2002; Nijman and Lenstra 2001). Y-chromosomal markers are especially relevant because hybridisation in species that live in herds occurs mostly via male introgressions. As a consequence Y-chromosomal alleles of the male lineage may have a broader geographical range than alleles informative for the female mitochondrial lineage (Lyrholm *et al.* 1999; Van Hooft 2001; Wang and Schreiber 2001). However, few Y-chromosomal markers are available (Edwards *et al.* 2000). A btDYZ-1/*Hae*III RFLP and the Y-chromosomal microsatellites INRA124 and INRA008 have been used in studies on the African bovine populations (Bradley *et al.* 1994; Hanotte *et al.* 2000; Van Hooft *et al.* 2002). Ward *et al*. (2001) described bison-specific alleles of the Y-chromosomal microsatellite BYM1.

Previously we have compared sequences of Y-chromosomal genes in order to investigate the phylogeny of the bovine species (Verkaar *et al.* 2003). Here we describe assays based on mutations in *SRY* (sex determining region Y-chromosome) and in the multicopy *TSPY* (testis-specific protein, Y-encoded, (Arnemann *et al.* 1991; Jakubiczka *et al.* 1993; Vogel *et al.* 1997). Detection of these mutations by convenient competitive PCR or restriction fragment length polymorphism (RFLP) analysis have been applied to the analysis of bulls from American bison populations in Belgium and to the paternal origins of Indonesian Bali and Madura cattle, respectively.

2. Material and Methods

2.1 Blood samples

Samples from American bison (*Bison bison*) were obtained from Artis Zoo, Amsterdam or La Ferme des Bisons, Recogne, Belgium. Wisent (*Bison bonasus*) and yak *(Bos grunniens* samples were obtained from Artis Zoo, banteng (*Bos javanicus*) from Blijdorp Zoo, Rotterdam, and taurine cattle (*Bos taurus*) from the Faculty of Veterinary Medicine, Utrecht. Blood samples from five Bali cattle (*Bos javanicus*) bulls and two Madura cattle (*Bos indicus*) bulls were collected at breeding stations (Malang, Indonesia). Two samples from zebu (*Bos indicus*) bulls were donated by Dr. D. G. Bradley (Trinity College, Dublin) and a sample from a beefalo bull by Dr. B. Morris (Stormont Laboratories, Woodland, USA). DNA was isolated from the whole blood by using standard SDS/proteinase K extraction (Sambrook *et al.* 1989), the GuITC protocol (Ciulla *et al.* 1988; Sambrook *et al.* 1989) or the Qiagen blood extraction method (Qiagen).

2.2 SRY and TSPY typing

The typings have been developed on the basis of recently submitted *SRY* nucleotide data from banteng (AY079146), bison (AY079141) and wisent (AY079142) respectively (Verkaar *et al.* 2003). Numbering of primer binding sites and restriction sites refer to the Genbank entry AB039748 for bovine *SRY* (Kato *et al.* 1995).

For differentiation of bison and cattle Y-chromosomes, PCR was performed in a total volume of 25 µl, containing 50 ng genomic DNA and 50 ng of primers SRY-1 (5'-GTT GAT GGG TTT GGG CTG ACT) and SRY-3 (5'- AAA TTG AGA TAA AGA GCG CCT) in Taq DNA polymerase buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP and 1.25 U Taq DNA polymerase (Promega, Madison-WI, USA). The program consisted of an initial denaturation of 2 min at 95°C followed by 30 cycles of 15 s at 92°C, 30s at 60°C and 45 s at 72°C and by a final extension of 5 min at 72°C. PCR products were fractionated on a 1% agarose/0.5 x TBE gel (SphaeroQ, Leiden, The Netherlands), excised and extracted with the QiaexII gel extraction kit (QIAGEN Inc., Valencia-CA, USA). Five µl of PCR product was digested by addition of 5 to 10 U of *Btr*I (New England BioLabs, Beverley-MA, USA)

and the recommended concentrated reaction buffer. Samples are digested for 3 h at 37°C and fractionated on a 2% agarose/0.5 x TBE gel.

For differentiation of bison and wisent, competitive PCR was performed as the normal PCR (see above) with primer SRY-3 (50 ng), SRY-1 (10 ng) and an internal primer SRY-bison (5'-ACA GCA ACA AAC TAC TCT CT, 40 ng). The nucleotide of this primer at the 3'end of SRY-bison only matches the sequence of American bison.

For detection of the banteng Y-chromosome, PCR was performed as described above with 50 ng of primers SRY-4 (5'-GCC TGG ACT TTC TTG TGC TTA) and SRY-5 (5'-ACA GTG GGA ACA AAA GAC TAT). After purification (see above) five µl of the PCR product was digested by addition of 5 to 10 U of *Bfa*I (New England BioLabs) and the recommended concentrated reaction buffer. Samples were digested for 1.5 h at 37°C and fractionated on a 2.5% agarose/0.5 x TBE gel. Sequencing was performed with 200 ng PCR product and a Cy5 Big Dye terminator kit (Applied Biosystems) on an ABI Prism 310 Sequencer.

Amplification of the *TSPY* multicopy genes was performed as described above with 50 ng of primers TSPY-L (5'-TCA TCA GCG AAG ACG TGT GGG, positions 2172-2192 of Genbank entry U75895, Vogel *et al.*, 1997) and 50 ng of TSPY-R (5'-AAG TGG CGA GAG CGT GTT TTG G, positions 3188-3123). For differentiation of cattle and zebu versus bison, wisent and yak, five µl of the purified PCR product was digested by addition of 2 to 5 U of *Rsa*I (Promega) and the recommended concentrated reaction buffer. Samples were digested for 1 h at 37°C and fractionated on a 1% agarose/0.5 x TBE gel.

3. Results

3.1 Differentiation of cattle and bison

Previously, it has been demonstrated that at least 6% of the American bisons carry mitochondria from taurine cattle (Ward *et al.* 2001). In a population of imported bisons in Belgium, we found by *Xba*I and *Hin*fI PCR-RFLP analysis (Verkaar *et al.* 2002) mitochondria of taurine origin in 2 out of 72 bisons (data not shown). In contrast, no taurine-specific alleles of the Y-chromosomal BYM1 microsatellite were found in American bisons

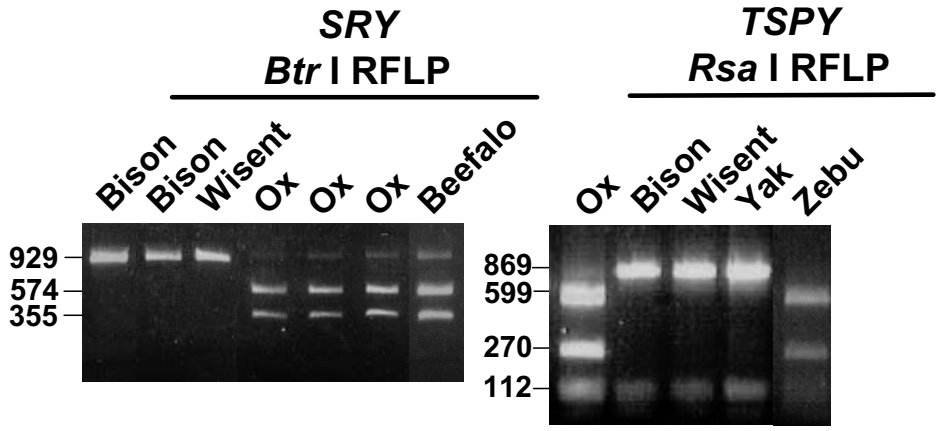

Figure 1. Differentiation of bison and bovine Y-chromosomes by PCR-RFLP analysis of *SRY* and *TSPY* from the indicated species

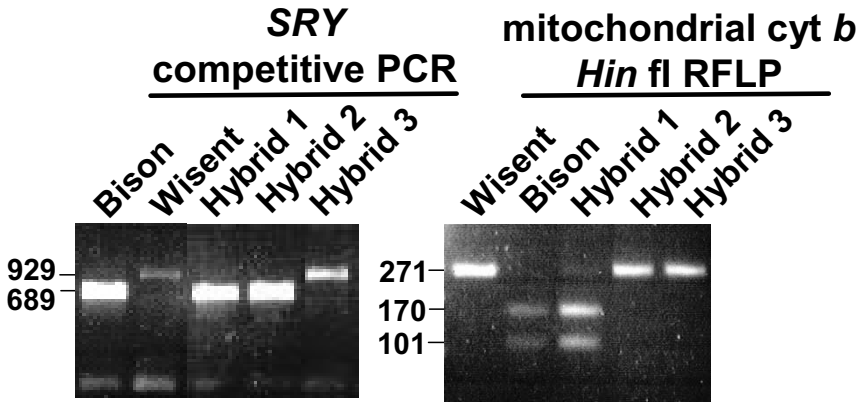

Figure 2. Differentiation of bison and wisent Y-chromosomes and mitochondrial DNA: Analysis of 3 individuals (hybrid 1, 2, 3, respectively) from a Belgian mixed wisent-bison population by competitive PCR of *SRY* and PCR-RFLP of the mitochondrial cytochrome *b* gene (Verkaar *et al.* 2002)

(Ward *et al.* 2001). However, the hybrid beefalo cattle are bred with bulls from both species.

We designed a convenient method for differentiation of the bison and taurine Y-chromosomes by PCR-RFLP analysis of a C→T mutation on position 1225 (numbering of AB039748; Verkaar *et al.*, 2003), which has led to the loss of a *Btr*I site in the *SRY* gene from American bison and wisent, but not in *SRY* from taurine cattle (Figure 1). *Btr*I analysis of a beefalo bull available for analysis suggested that this animal descends from a taurine or zebu bull (Figure 1).

An alternative Y-chromosomal PCR-RFLP assay may be based on a G→A mutation on position 2916 (Genbank entry U75895, Vogel *et al.*, 1997) of the multicopy *TSPY* gene. This mutation abolishes an *Rsa*I site and distinguishes American bison, wisent and yak (fragments of 36, 112 and 870 bp) from taurine cattle and zebu (fragments of 36, 112, 271 and 599 bp; Figure 1).

3.2 Differentiation of American bison and wisent

The wisent was almost extinct in 1922 (Felius 1985; Lenstra and Bradley 1999). Essential in the revival program, which started with 22 bulls and 22 cows, was the protection against introgression of American bison. The Y-chromosomes from bison and wisent can be distinguished by a G→T mutation at position 1130 of *SRY* (Genbank entry AB039748). Since this did not create or abolish a restriction site, we designed a competitive PCR in which the forward primer competes with a third, bison-specific primer (SRY-bison) with at its 3' end the diagnostic mutation. By varying the primer concentration we found that unambiguous differentiation of bison and wisent was obtained by a 1:4:5 ratio of the concentrations of the forward primer, internal forward primer and reverse primer, respectively. This generates with wisent the full-length product (SRY-1 to SRY-3, 929 bp) and with bison a predominant shorter product (SRY-3 to SRY-bison, 689 bp). Competitive PCR with several samples of taurine cattle (3), yak (4), wisent (3) and American bison (6) yielded the 689-bp fragment only with bison (results for American bison and wisent shown in Fig. 2).

We used this assay in combination with a mitochondrial test on the maternal descendance (Verkaar *et al.* 2002) of three bulls from a privately held bison herd in Halle, Belgium. As evident from Fig. 2, bison 1 descends paternally and maternally from bison,

bison 2 has a maternal wisent and a paternal bison origin, respectively, and bison 3 descends both paternally and maternally from wisent. This suggests that, in contrast to the differential taurine introgression in bison (Ward *et al.* 2001), cross-breeding in mixed populations of American bisons and wisents is symmetrical.

3.3 Differentiation of zebu and banteng.

The predominant cattle species in Indonesia is the zebu (*Bos indicus*), which is reported to carry mitochondrial DNA of the Banteng type (Kikkawa *et al.* 2003). Bali cattle descends from the wild banteng (*Bos javanicus*), although zebu introgression has been suspected (Namikawa 1981). Bali cattle bred outside their region of origin are clearly of hybrid origin, as shown by the analysis of mitochondrial DNA, AFLP, SFLP and microsatellite analysis of Malaysian Bali cattle (Nijman *et al.* 2002). Madura cattle has the typical zebu hump, but is also supposed to result from zebu-banteng hybridization (Payne and Rollinson 1976). The hybrid origin of the Madura nuclear genome has been verified by SFLP and AFLP analysis, while the mitochondrial DNA was either of zebu or banteng origin (Nijman *et al.* 2002).

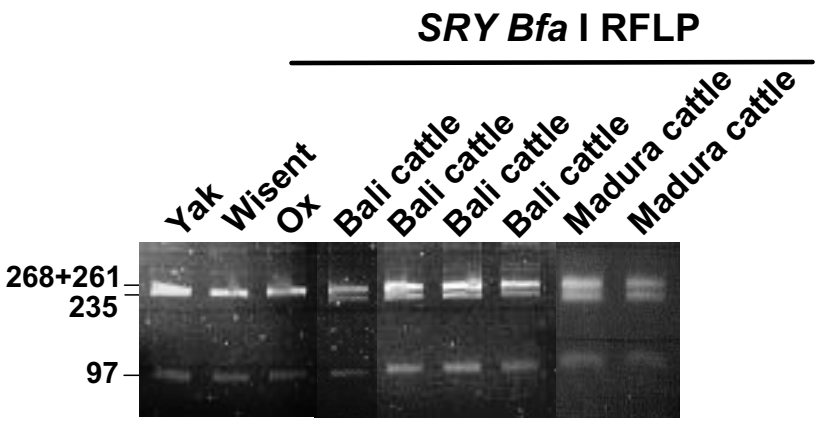

Figure 3. *Bfa*I PCR-RFLP of *SRY* from the indicated bovine species, Bali-cattle (4 animals) and Madura cattle (2 animals), respectively. In separate experiments it was checked that the nucleotide sequences as well as the corresponding *Bfa*I cleavage patterns of banteng and Bali cattle were identical and different from other bovine species

However, no data are available yet about parental lineages in Bali and Madura cattle. The Y-chromosome from banteng can be detected via an A→G mutation at position 2018 of database entry AB039748, which creates an extra *Bfa*I site. PCR-RFLP with *Bfa*I bovini other than banteng yield fragments of 270, 261 and 97 bp respectively, while in banteng, the 261 bp fragment is cleaved in a 235 and a 26 bp fragment (representative results in Figure 3).

We analyzed five Bali bulls from breeding farms with the *Bfa*I PCR-RFLP Y-chromosomal test. As shown in Figure 3 for 4 of the animals, the Bali cattle bulls appeared to descend from banteng bulls. In addition, a mitochondrial PCR-RFLP test (Verkaar *et al.* 2002) showed also that the maternal lineage is also of banteng origin. Interestingly, the two Madura bulls that were available for analysis also carried banteng Y-chromosomes (Figure 1).

These results suggest that in combination with typing of nuclear and mitochondrial DNA (Nijman *et al.* 2003; Verkaar *et al.* 2002) Y-chromosomal assays are a useful tool for the conservation of the original Indonesian cattle breeds.

4. Discussion

In this paper we have used sequence variation in the Y-chromosomal *SRY* gene for the design of convenient tests of paternal lineages in bovine populations (Table 1). *SRY* is the most obvious target for these assays, since it is the only single copy gene without a X-chromosomal homologue. As shown in Figure 1, the concerted evolution of the multi-copy *TSPY* gene allows an alternative assay for the origin of paternal lineages (Tosi *et al.* 2000). In addition to the tests described in this paper, similar tests can be designed for the detection of taurine or yak Y-chromosomes (Table 1). Differentiation of taurine and indicine Y-chromosomes by a *Tru*9I digestion would provide a convenient way of tracing the paternal origin of cattle populations (Bradley *et al.* 1994; Hanotte *et al.* 2000; Kikkawa *et al.* 2003).

Previously described tests were based on Y-chromosomal microsatellites. However, this requires radiochemical or fluorescent labels as well as sequencing equipment. Furthermore, microsatellites are only informative for the species origin if species-specific

Table 1. Diaganostic restriction sites in the Y-chromosomal *SRY* gene. The numbering refers to Genbank entry AB039748 of the taurine *SRY* (Kato *et al.* 1995). Bold printing indicates sites used in this study

Position	Restriction enzyme	Species having this site in *SRY*
1009	*Mae*I	yak
1225	*Btr*I	ox, zebu, gayal, banteng, yak
1708	*Tru*91	zebu, gayal, banteng, yak, bison, wisent
1843	*Mae*I	yak
2016	*Bfa*I	banteng

alleles have been fixed (Edwards *et al.* 2000). It may be noteworthy that wisent did not have the short bison-like alleles of the BYM1 marker, illustrating the dynamic evolution of microsatellites (results not shown). In contrast, the sequence variants of the *SRY* gene are stable and several methods are available for their detection. PCR-RFLP and competitive PCR require modest equipment and is suitable for low-throughput implementations in zoos and wildlife-management institutes. With appropriate controls it can be checked if the enzymatic digestion has been successful. If more single-copy Y-chromosomal sequences are becoming available (Hellborg and Ellegren 2003), assignments can be based on more than one mutation as safeguard against intraspecies polymorphisms. Although generally Y-chromosomal polymorphisms are scarce, the mutation in the *SRY* gene of yak on position 1292 observed by Kikkawa *et al.* (2003) was not observed in the yak bulls sampled by us (Verkaar *et al.* 2003).

Uniparentally inherited markers may detect the origin of a population even if many generations of breeding has obscured the original species composition (Bradley *et al.* 1994; Ward *et al.* 2001). Since the female calves normally stay in the herd where they were born, the mitochondrial DNA sequence reveals the species origin of the herd. In mammalian species, females are often philopatric, while the males cover a broader geographic range (Nijman *et al.* 2002). So Y-chromosomal markers may be expected to have another geographical distribution than the mitochondrial markers. In addition, paternally inherited

markers may reveal male introgression via contacts of the herd with species from neighboring habitats. If introgression has been systematic, for instance upgrading of a breed, Y-chromosomal markers will correlate with the phenotypic variation and autosomal markers.

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References

Arnemann, J., S. Jakubiczka, S. Thuring and J. Schmidtke, 1991 Cloning and sequence analysis of a human Y-chromosome-derived, testicular cDNA, TSPY. Genomics **11:** 108-114.

Bongso, T. A., M. Hilmi, M. Sopian and S. Zulkifli, 1988 Chromosomes of gaur cross domestic cattle hybrids. Res Vet Sci **44:** 251-254.

Bradley, D. G., D. E. MacHugh, P. Cunningham and R. T. Loftus, 1996 Mitochondrial diversity and the origins of African and European cattle. Proc Natl Acad Sci U S A **93:** 5131-5135.

Bradley, D. G., D. E. MacHugh, R. T. Loftus, R. S. Sow, C. H. Hoste *et al.*, 1994 Zebu-taurine variation in Y chromosomal DNA: a sensitive assay for genetic introgression in west African trypanotolerant cattle populations. Anim Genet **25:** 7-12.

Buntjer, J. B., M. Otsen, I. J. Nijman, M. T. Kuiper and J. A. Lenstra, 2002 Phylogeny of bovine species based on AFLP fingerprinting. Heredity **88:** 46-51.

Ciulla, T. A., R. M. Sklar and S. L. Hauser, 1988 A simple method for DNA purification from peripheral blood. Anal Biochem **174:** 485-488.

Edwards, C. J., C. Gaillard, D. G. Bradley and D. E. MacHugh, 2000 Y-specific microsatellite polymorphisms in a range of bovid species. Anim Genet **31:** 127-130.

Felius, M., 1985 Cattle breeds, an encyclopedia. Misset, Doetinchem, The Netherlands.

Hanotte, O., C. L. Tawah, D. G. Bradley, M. Okomo, Y. Verjee *et al.*, 2000 Geographic distribution and frequency of a taurine Bos taurus and an indicine Bos indicus Y specific allele amongst sub-saharan African cattle breeds. Mol Ecol **9:** 387-396.

Hellborg, L., and H. Ellegren, 2003 Y chromosome conserved anchored tagged sequences (YCATS) for the analysis of mammalian male-specific DNA. Mol Ecol **12:** 283-291.

Jakubiczka, S., F. Schnieders and J. Schmidtke, 1993 A bovine homologue of the human TSPY gene [published erratum appears in Genomics 1994 Jan 1;19(1):198]. Genomics **17:** 732-735.

Janecek, L. L., R. L. Honeycutt, R. M. Adkins and S. K. Davis, 1996 Mitochondrial gene sequences and the molecular systematics of the artiodactyl subfamily bovinae. Mol Phylogenet Evol **6:** 107-119.

Kato, Y., S. Sato, X. Cui, Y. Itagaki and S. Sutou, 1995 Cloning and characterization of bovine SRY gene. Animal Sci. Technol. **66:** 994-1000.

Kikkawa, Y., T. Takada, Sutopo, K. Nomura, T. Namikawa *et al.*, 2003 Phylogenies using mtDNA and SRY provide evidence for male-mediated introgression in Asian domestic cattle. Anim Genet **34:** 96-101.

Lenstra, J. A., and D. G. Bradley, 1999 Systematics and phylogeny of cattle. CAB Intl. Ed. by R. Fries and A. Ruvenski**:** 1-14.

Lyrholm, T., O. Leimar, B. Johanneson and U. Gyllensten, 1999 Sex-biased dispersal in sperm whales: contrasting mitochondrial and nuclear genetic structure of global populations. Proc R Soc Lond B Biol Sci **266:** 347-354.

Namikawa, T., 1981 Geographic distribution of bovine haemoglobin-beta (Hbb) alleles and the phylogenetic analysis of cattle in Eastern Asia. Z. Tierzüch. Zuchtungsbiol. **98:** 151-159.

Nijman, I. J., D. G. Bradley, O. Hanotte, M. Otsen and J. A. Lenstra, 1999 Satellite DNA polymorphisms and AFLP correlate with Bos indicus-taurus hybridization. Anim Genet **30:** 265-273.

Nijman, I. J., and J. A. Lenstra, 2001 Mutation and recombination in cattle satellite DNA: a feedback model for the evolution of satellite DNA repeats. J Mol Evol **52:** 361-371.

Nijman, I. J., M. Otsen, E. L. Verkaar, C. De Ruijter, E. Hanekamp *et al.*, 2003 Hybridization of banteng (Bos javanicus) and zebu (Bos indicus) revealed by mitochondrial DNA, satellite DNA, AFLP and microsatellites. Heredity **90:** 10-16.

Nijman, I. J., M. Otsen, E. L. C. Verkaar, C. de Ruijter, E. Hanekamp *et al.*, 2002 Hybridization of banteng (Bos javanicus) and zebu (Bos indicus) revealed by mitochondrial DNA, satellite DNA, AFLP and microsatellites. in press.

Payne, W. J. A., and D. H. L. Rollinson, 1976 Madura cattle. Z. Tierzüch Züchtsbiol. **93:** 89-100.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 Molecular cloning. A laboratory manual. Cold spring harbor laboratory press, Cold spring harbor, USA.

Tosi, A. J., J. C. Morales and D. J. Melnick, 2000 Comparison of Y Chromosome and mtDNA Phylogenies Leads to Unique Inferences of Macaque Evolutionary History. Mol Phylogenet Evol **17:** 133-144.

Van Hooft, P., 2001 Population genetics of the African buffalo. Thesis Wageningen University, Department of Environmental Sciences, Tropical Nature conservationand vertebrate ecology group**:** 61-83.

Van Hooft, W. F., A. F. Groen and H. H. Prins, 2002 Phylogeography of the African buffalo based on mitochondrial and Y-chromosomal loci: Pleistocene origin and population expansion of the Cape buffalo subspecies. Mol Ecol **11:** 267-279.

Verkaar, E. L. C., I. J. Nijman, M. Beeke, E. Hanekamp and J. A. Lenstra, 2003 Maternal and paternal lineages in cross-breeding bovine species: The unusual phylogeny of wisent. submitted.

Verkaar, E. L. C., I. J. Nijman, K. Boutaga and J. A. Lenstra, 2002 Differentiation of cattle species in beef by PCR-RFLP of mitochondrial and satellite DNA. Meat Science **60:** 365-369.

Vogel, T., F. Dechend, E. Manz, C. Jung, S. Jakubiczka *et al.*, 1997 Organization and expression of bovine TSPY. Mamm Genome **8:** 491-496.

Wang, M., and A. Schreiber, 2001 The impact of habitat fragmentation and social structure on the population genetics of roe deer (Capreolus capreolus L.) in Central Europe. Heredity **86:** 703-715.

Ward, T. J., J. P. Bielawski, S. K. Davis, J. W. Templeton and J. N. Derr, 1999 Identifiction of domestic cattle hybrids in wild cattle and bison species: a general approach using mtDNA markers and the parametric bootstrap. Animal Conservation**:** 51-57.

Ward, T. J., L. C. Skow, D. S. Gallagher, R. D. Schnabel, C. A. Nall *et al.*, 2001 Differential introgression of uniparentally inherited markers in bison populations with hybrid ancestries. Anim Genet **32:** 89-91.

CHAPTER 2C

Ruminant DNA detection: Real-time PCR Detection of Ruminant DNA

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Abstract

In order to control the spread of transmissible spongiform encephalopathy (TSE), several DNA methods have been described for the detection of the species origin of meat-and-bone-meal. Most of these methods are based on the amplification of a mitochondrial DNA segment. We have developed a semi-quantitative method based on real-time PCR for detection of ruminant DNA, targeting an 88-bp segment of the ruminant short interspersed nuclear element (SINE) Bov-A2. This method is specific for ruminants and is able to detect as little as 10 fg of bovine DNA. Autoclaving decreased the amount of detectable DNA, but positive signals were observed in feedstuff containing 10% bovine material if this had not been rendered in accordance with the regulations, i.e. heated at 134°C for 3 instead of 20 minutes.

1. Introduction

The use of meat and bone meals (MBM) from sheep in feedstuff for cattle has been implicated in the emergence of BSE (bovine spongiform encephalopathy) (Taylor and Woodgate 1997). Human consumption of beef from BSE-infected cattle has caused the emergence of a juvenile form of Creutzfeldt-Jakob's disease (CJD) in man, the new variant CJD (vCJD) (Will 1999). After the 1988 UK ban on feeding ruminant material to other ruminants, European guidelines now prohibit with a few exceptions the addition of any processed animal material to feedstuff unless the absence of ruminant proteins can be demonstrated (EEC Commission decision 94/381/EEC). In order to enforce this, several tests have been developed to detect animal material in feedstuff. Currently, the official method in the European Union is the detection of animal bone fragments by microscopy. This method, however, cannot distinguish bone fragments in MBM if these are finely grinded. Furthermore, this method does not allow identification of the species and requires trained personnel. Commercially available ELISA kits detect species-specific antigens, but are only partially adequate for samples that have been subjected to a heat treatment (Von Holst et al. 2000).

Because of its heat stability, DNA is a suitable target for species identification assays. Dot-spot hybridization is suitable for detection of species-specific DNA repeats in

heated or even autoclaved meat samples (Buntjer *et al.* 1999; Buntjer *et al.* 1995), but does not have the sensitivity required for analysis of feedstuff (unpublished results). PCR-based methods are more sensitive and several protocols are available for the extraction and analysis of DNA from feedstuff (Table 1). Most of these are based on the detection of species-specific mitochondrial DNA sequences and determine only the presence of bovine material (Kingombe *et al.* 2001; Krcmar and Rencova 2001; Lahiff *et al.* 2002; Tartaglia *et al.* 1998), use generic primers (Kocher *et al.* 1989; Meyer and Candrian 1996) that do not perform equally well in all species (Verkaar *et al.* 2001) or yield only qualitative results. Two recently developed methods (Tajima *et al.* 2002; Walker *et al.* 2003) are targeted to SINE repetitive elements specific for the genomes of ruminants, pigs and poultry, respectively.

The amplification of these SINE sequences and of a bovine-specific satellite DNA sequence has been combined with real-time detection by measuring SYBR Green fluorescence (Walker *et al.* 2003). However, SINE elements are heterogeneous (Lenstra *et al.* 1993) and the design of PCR primers that match a significant proportion of the SINE copies may not be straightforward.

Detection of ruminant material in the food chain is especially relevant because of the prevalence of transmissible spongiform encephalopathy in at least three ruminant species and the infectivity of abnormal bovine prions in men (Dalton and Check 2002; Hamir *et al.* 2001; Taylor and Woodgate 1997; Will 1999). As a further refinement of a quantitative and specific detection of ruminant DNA, we describe here a 5'-nuclease or Taqman® assay based on the relatively homogeneous Bov-A2 SINE element (Lenstra *et al.* 1993). With this method, real-time fluorescent detection is generated by the hydrolysis of a third, internal oligonucleotide, which increases the specificity relative to a normal two-primer PCR. An alignment of Bov-A2 elements allowed the selection of the primers and the Taqman probe in the most conserved region of the SINE sequence, while the sensitivity is further optimized by reducing the size of the amplicon to 88 bp. The sensitivity and specificity of this method as well as results with experimental MBM and feedstuff samples is described.

2. Materials and methods

2.1 Samples and DNA extraction

Genomic DNA was isolated from blood samples by using the Qiagen blood extraction kit (Qiagen Inc., Valencia-CA, USA) or by GuITC (guanidium-isothiocyanate) extraction (Ciulla *et al.* 1988; Sambrook *et al.* 1989)**.** DNA concentrations were determined spectrophotometrically. Experimental MBM samples provided by the Institute of Animal Science and Health (ID-DLO, Lelystad) contained 90% predominantly porcine material and 10% bovine brain material. The samples have been exposed to various heat treatment cycles performed in an autoclave with internal monitoring conditions. Feedstuff samples prepared by Labocor (Madrid) contained equal amounts of maize, soya, barley and wheat with commercial MBM at quantities ranging from 0.1 to 10 %. DNA from 200 mg of feedstuff was extracted with the Wizard Magnetic DNA purification system for food (PROMEGA, Madison WI, USA).

2.2 Quantitative PCR of the Bov-A2 segment

Primers and an internal probe specific for the ruminant Bov-A2 SINE consensus sequence Genbank entry X64126 (Lenstra *et al.* 1993) were designed using the Primer Express program (Applied Biosystems, Foster City-CA, USA). Primers (5'-GAC TGA GCG ACT TCA CTT TCA) and (5'-GGA TTC TCC AGG CAA GAA CA) amplify a fragment of 88 bp (position 104—191 in Genbank entry X64126). The 5'-nuclease Taqman® probe (5'-FAM-5'-TTG GAG AAG GAA ATG GCA ACC CAC TCC-TAMRA-3') was synthesized by Eurogentec (Seraing, Belgium). Real-time PCR has been performed in a total volume of 25 µl, containing 12.5 µl Taqman Mastermix (Applied Biosystems), 0.45 mM of both primers and 0.2 mM probe. The program included 10 min predenaturation at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. PCR amplification and detection was performed on a Taqman 7000 instrument (Applied Biosystems).

Table 1. DNA based assays of feedstuff

Reference	Target	Amplicon length	Detection method
Krcmar and Rencova 2001; Myer et al. 2001; Tartaglia et al. 1998; Wang et al. 2000	Bovine ATPase subunits 6 and 8	271	PCR, electrophoresis
Frezza et al., 2003	Bovine ATPase subunits 6 and 8	271, 240, 147	Competitive PCR, electrophoresis
Lahiff et al., 2002	Bovine ATPase 8 subunit	271	PCR, online 5' nuclease assay
Bellagamba et al., 2001	Vertebrate mitochondrial cytochrome b	359	PCR-RFLP
Bellagamba et al., 2003	Ruminant mitochondrial 12 S rRNA gene	231	PCR, agarose gel electrophoresis
	Pig mitochondrial 12 S rRNA gene	186	
	Poultry mitochondrial 12 S rRNA gene	256	
Kingombe et al. 2001	Vertebrate mt $tRNA^{Glu}$-cytochrome b	464	PCR, electrophoresis
	bovine mtDNA	274	"
Colgan et al. 2001; Lahiff et al. 2001	Bovine ATPase 8 subunit	271	PCR, electrophoresis
	Ovine ATPase 8 subunit	225	
	Porcine ATPase 8 subunit	212	"
	Chicken ATPase 8 subunit	266	"
Tajima et al. 2002	Ruminant Bov-B SINE	181	PCR, electrophoresis
	Porcine PRE-1 SINE	179	"
	Avian CR-1 SINE	201	"
Walker et al. 2003	Bovine satellite 1.711B	98	PCR, online SYBR green-fluorescence
	Porcine PRE-1 SINE	134	
	Avian CR1 SINE	169	
	Ruminant Bov-tA SINE	100	
This paper	Ruminant Bov-A2 SINE	88	PCR, online 5' nuclease assay

3. Results

For the development of a ruminant-specific quantitative PCR assay the Bov-A2 SINE repeat was chosen as target because it is more homogeneous than other major SINE sequences Bov-tA and Bov-B (Lenstra *et al.* 1993). Primers and probe were designed by following the guidelines of Applied Biosystems in Primer Express and were located in the most conserved part of Bov-A2. A BLAST search showed that the forward primer, the reverse primer and the Taqman probe had 54, 97 and 97 perfect matches in 13.4 Mb of bovine DNA and 5, 14 and 9 perfect matches in 1.9 Mb of ovine DNA, respectively. The optimal concentrations were found to be 0.45 mM for both primers and 0.2 mM for the probe.

Fig. 1 shows the analysis of tenfold dilutions of bovine genomic DNA. Although the Ct value is less accurate at the lowest DNA concentration, 8.5 fg bovine DNA still gave signals significantly higher than the blank value. Plots of Ct values versus the logarithm of the DNA concentration (Fig. 2) show good linearity ($R^2 = 0.995$). Essentially the same correlation between DNA concentrations and Ct values was obtained with sheep and goat DNA, respectively. DNA from other ruminants (giraffe, mule deer) also generated clearly positive signals (Table 2). With DNA from various non-ruminant species, Ct values were 25 or higher (Table 2) and probably correspond to traces of bovine DNA in the sample.

The scrapie-agent is capable of withstanding temperatures of up to 137°C (Brown *et al.* 2003). We investigated detection limits of bovine DNA in samples of feedstuff mixed with 10% bovine brain tissue subjected to different heat treatments (Table 3). Bovine DNA could still be detected after 3 min at 134°C, but the amount of DNA decreased by a longer heat treatment (20 min at 133°C) or by heating at a higher temperature (3 min at 140°C). However, even after treatment under these extreme conditions 5 to 18 pg DNA could still be detected. In commercial feedstuff samples containing MBM only 4 to 70 fg ruminant DNA was measured (data not shown), which is around the detection limit.

Table 2. Specificity of the BovA detection

Species	Amount of DNA (ng)	Ct value
Mule deer	125	11.5
Giraffe	40	13.1
Pig	12.5	27.7
Horse	12.5	29.2
Chicken	12.5	31.0
Turkey	12.5	32.1

Table 3. Ruminant DNA detected in 200 mg feed stuff samples containing 10% bovine brain material and subjected to the indicated heat treatment. Amounts of DNA were read from a calibration curve as in Figure 1 based on the analysis of 850 pg to 85 fg bovine genomic DNA (Ct 14.1 to 27.3, 31.2 for blank sample) in the same run

Heat treatment	DNA (pg)
100°C, 20 min	1082
105°C, 3 min	255
121°C, 3 min	777
121°C, 3 min	255
121°C, 15 min	527
134°C, 3 min	270
134°C, 3 min	249
133°C, 20 min	5.2
133°C, 20 min	1.7
140°C, 3 min	17.6
140°C, 3 min	5.0

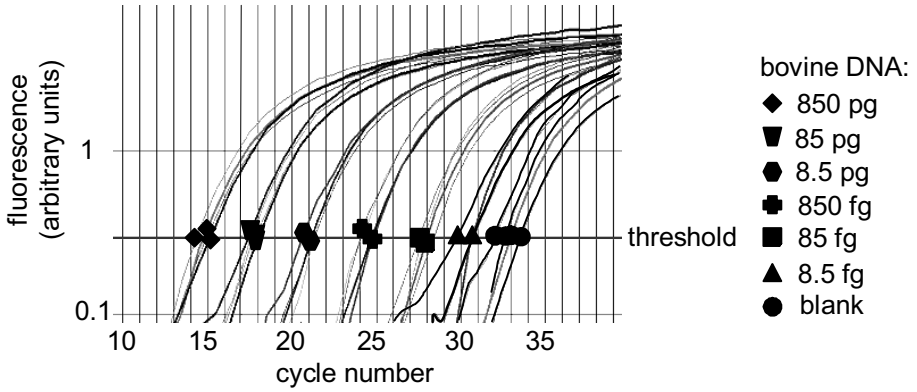

Figure 1. Fluorescent profiles of the PCR reaction with tenfold dilutions of genomic bovine DNA and a blank

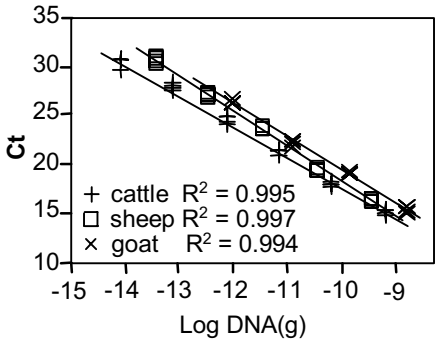

Figure 2. Ct values (cycle number at which the fluorescence passes the threshold, see Fig 1) *vs* the logarithm of the amount of bovine genomic DNA (g) and corresponding regression data

4. Discussion

Growing evidence for causal relations of BSE in cattle and vCJD (Will 1999) has led to strict regulations on the composition of feedstuff and a requirement of test methods in order to be able to enforce the rules (Taylor and Woodgate 1997). Current EU legislation prohibits with a few exceptions the addition of any animal material. Most assays of feedstuff described so far (Table 1) test only for bovine material and will not detect material from other ruminants like sheep and deer, which are potentially infected with scrapie or chronic wasting disease (CWD), respectively (Dalton and Check 2002; Hamir *et al.* 2001).

Ideally, a test should have a well-defined and relevant species-specificity, a linear response, an adequate sensitivity for degraded DNA, and a high throughput.

We have developed a real-time PCR assay for the presence of the ruminant-specific Bov-A2 SINE, which is estimated to account for 1.8% of the bovine genome (Lenstra *et al.* 1993). SINE elements have a broader taxonomic range than the species-specific centromeric satellite repeats (Jobse *et al.* 1995). As a consequence, SINE sequences are heterogeneous and primers may match to only few genomic SINE copies. For instance, one of the primers designed for the amplification of the more degenerate Bov-tA elements (Walker *et al.* 2003) has only few complete matches to bovine or ovine SINE sequences in the nucleotide database and the second primer has no complete match at all. The Bov-A2 SINE is more homogeneous than the related Bov-tA and Bov-B elements (Lenstra *et al.* 1993) and is present in comparable amounts in the genomes of all ruminants (Buntjer *et al.* 1997; Jobse *et al.* 1995; Nijman *et al.* 2002). Our primers have been optimized for cattle, but detect also DNA from sheep, goat, deer and giraffe.

The specificity of our test has further been enhanced relative to gel electrophoresis or SYBR-Green detection (see Table 1) by the use of a third target-specific oligonucleotide in a 5'-nuclease detection. This may be essential for critical samples and also allows a multicolor multiplex detection (Walker *et al.* 2003). The FAM-TAMRA probe used in this study matches a conserved region and has several matches to ruminant SINE elements in the nucleotide database.

With cattle DNA the signal was found to be linear over a million fold range of DNA concentrations with essentially the same response with sheep and goat DNA, respectively.

The detection limit of 10 fg compares favorably with the detection of 1 pg achieved by competitive PCR (Frezza *et al.* 2003) or of 0.1 pg achieved by on-line PCR of the Bov-tA SINE with SYBR detection (Walker *et al.* 2003) and is in the same range as the 18.75 fg reported for a Bov-B SINE PCR (Tajima *et al.* 2002). An important factor that in practice may limit the sensitivity is contamination with bovine DNA, for instance during sample processing or by the frequent use of bovine serum albumin in biochemical reagents.

The sensitivity for degraded DNA samples was optimized by a short amplicon length. Analysis of a panel of experimental MBM samples containing 10% bovine brain material indicated that bovine DNA can be detected even after heating at 134°C for 3 min. The signal of DNA detected after 20 min at 133°C or 3 min at 140°C corresponds to 5 to 18 pg DNA. These values probably indicate a contamination of the experimental MBM samples after the heat treatment, since lower values were measured in commercial MBM or in the blank sample.

An attractive feature of the on-line detection is that no post-PCR steps are required and that the method is suitable for automated high-throughput analysis. Although a further validation with experimental and practical feedingstuff samples is required, there is proof-of-concept of the quantitative assay of the Bov-A2 SINE or other repetitive elements (Tajima *et al.* 2002; Walker *et al.* 2003) as tools for the detection of the origin of feedstuff and for the control of the spread of transmissible encephalopathic diseases.

Acknowledgements

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References

Brown, P., R. Meyer, F. Cardone and M. Pocchiari, 2003 Ultra-high-pressure inactivation of prion infectivity in processed meat: a practical method to prevent human infection. Proc Natl Acad Sci U S A **100:** 6093-6097.

Buntjer, J. B., I. A. Hoff and J. A. Lenstra, 1997 Artiodactyl interspersed DNA repeats in cetacean genomes. J Mol Evol **45:** 66-69.

Buntjer, J. B., A. Lamine, N. Haagsma and J. A. Lenstra, 1999 Species identification by oligonucleotide hybridisation: the influence of processing of meat products. Jounal of the Science of Food and Agriculture**:** 53-57.

Buntjer, J. B., J. A. Lenstra and N. Haagsma, 1995 Rapid species identification in meat by using satellite DNA probes. Z Lebensm Unters Forsch **201:** 577-582.

Ciulla, T. A., R. M. Sklar and S. L. Hauser, 1988 A simple method for DNA purification from peripheral blood. Anal Biochem **174:** 485-488.

Dalton, R., and E. Check, 2002 Prion research stepped up as fear grows of deer disease. Nature **419:** 236.

Frezza, D., M. Favaro, G. Vaccari, C. von-Holst, V. Giambra *et al.*, 2003 A competitive polymerase chain reaction-based approach for the identification and semiquantification of mitochondrial DNA in differently heat-treated bovine meat and bone meal. J Food Prot **66:** 103-109.

Hamir, A. N., R. C. Cutlip, J. M. Miller, E. S. Williams, M. J. Stack *et al.*, 2001 Preliminary findings on the experimental transmission of chronic wasting disease agent of mule deer to cattle. J Vet Diagn Invest **13:** 91-96.

Jobse, C., J. B. Buntjer, N. Haagsma, H. J. Breukelman, J. J. Beintema *et al.*, 1995 Evolution and recombination of bovine DNA repeats. J Mol Evol **41:** 277-283.

Kingombe, C. I. B., E. Luthi, H. Schlosser, D. Howald, M. Kuhn *et al.*, 2001 A PCR-test for species-specific determination of heat treatment conditions of animal meals as an effective prophylactic method for bovine spongiform encephalopathy. Meat Science**:** 35-41.

Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Paabo *et al.*, 1989 Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci U S A **86:** 6196-6200.

Krcmar, P., and E. Rencova, 2001 Identification of bovine-specific DNA in feedstuffs. J Food Prot **64:** 117-119.

Lahiff, S., M. Glennon, J. Lyng, T. Smith, N. Shilton *et al.*, 2002 Real-time polymerase chain reaction detection of bovine DNA in meat and bone meal samples. J Food Prot **65:** 1158-1165.

Lenstra, J. A., J. A. van Boxtel, K. A. Zwaagstra and M. Schwerin, 1993 Short interspersed nuclear element (SINE) sequences of the Bovidae. Anim Genet **24:** 33-39.

Meyer, R., and U. Candrian, 1996 PCR bases DNA analysis for the identification and characterization of food components. Lebensm.wiss. u. Technol.**:** 1-9.

Nijman, I. J., P. van Tessel and J. A. Lenstra, 2002 SINE Retrotransposition During the Evolution of the Pecoran Ruminants. J Mol Evol **54:** 9-16.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 Molecular cloning. A laboratory manual. Cold spring harbor laboratory press, Cold spring harbor, USA.

Tajima, K., O. Enishi, M. Amari, M. Mitsumori, H. Kajikawa *et al.*, 2002 PCR detection of DNAs of animal origin in feed by primers based on sequences of short and long interspersed repetitive elements. Biosci Biotechnol Biochem **66:** 2247-2250.

Tartaglia, M., E. Saulle, S. Pestalozza, L. Morelli, G. Antonucci *et al.*, 1998 Detection of bovine mitochondrial DNA in ruminant feeds: a molecular approach to test for the presence of bovine-derived materials. J Food Prot **61:** 513-518.

Taylor, D. M., and S. L. Woodgate, 1997 Bovine spongiform encephalopathy: the causal role of ruminant-derived protein in cattle diets. Rev Sci Tech **16:** 187-198.

Verkaar, E. L. C., I. J. Nijman, K. Boutaga and J. A. Lenstra, 2001 Differentiation of cattle species in beef by PCR-RFLP of mitochondrial and satellite DNA. Meat Science **60:** 365-369.

Von Holst, C., K. O. Honikel, W. Unglaud, G. Krame and E. Anklam, 2000 Determination of an appropriate heat treatment of animal waste using the ELISA technique: results of a validation survey. Meat Science **54:** 1-7.

Walker, J. A., D. A. Hughes, B. A. Anders, J. Shewale, S. K. Sinha *et al.*, 2003 Quantitative intra-short interspersed element PCR for species-specific DNA identification. Anal Biochem **316:** 259-269.

Will, R. G., 1999 The transmission of prions to humans. Acta Paediatr Suppl **88:** 28-32.

CHAPTER 3

Maternal and paternal lineages in crossbreeding bovine species. Has the wisent a hybrid origin?

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Abstract

The tribe *Bovini* comprises cattle and cattle-like species. Reconstructions of their phylogeny have so far been incomplete and do not indicate a close relationship of American bison and wisent (European bison). We have compared the sequences of three mitochondrial and two Y-chromosomal DNA segments. Mitochondrial DNA indicates that four distinct maternal lineages diverged after an early split-off of the buffalo species, leading to (1) taurine cattle and zebu, (2) wisent, (3) American bison and yak, and (4) banteng, gaur and gayal, respectively. At a higher level, lineages (1) and (2) and lineages (3) and (4) are probably associated. In contrast, Y-chromosomal sequences indicate a close association of American and European bison, which is in agreement with their morphological similarity, complete fertility of hybrid offspring and AFLP fingerprints of nuclear DNA. One explanation for the anomalous divergence of the mitochondrial DNA from the two bison species is lineage sorting, which implies that two distinct mitochondrial lineages coexisted in the bison-yak branch until the recent divergence of American bison and wisent.
Alternatively, the wisent may have emerged by species hybridization initiated by introgression of bison bulls in another ancestral species. This 'transpatric' mode of species formation would be consistent with the recent appearance of the wisent in the fossil record without clearly identifiable ancestors.

1. Introduction

The tribe *Bovini* comprises the bovine species, several of which have been domesticated as cattle (Lenstra and Bradley 1999). Within this taxon, the most early divergence 5 to 10 Myr ago separated water buffalo (*Bubalus bubalis*), anoa *(Bubalus depressicornis*) and African buffalo (*Syncerus caffer*) from the *Bos* and *Bison* species (Buntjer *et al.* 2002; Hassanin and Douzery 1999a; Hassanin and Douzery 1999b; Janecek *et al.* 1996). Speciation of the latter species after a divergence time of about 1 Myr has not been complete, since all female hybrid offspring as well as the male hybrids resulting from ox-zebu and bison-wisent cross-

ings are fertile. Hybridization of bovine species is either spontaneous or by organized crossing. (Lenstra and Bradley 1999; Nijman *et al.* 1999; Ward *et al.* 1999).

So far the bovine phylogeny has not been resolved. Morphological features (Bohlken 1961; Geraads 1992; Groves 1981) and nuclear gene sequences (Chikuni *et al.* 1995) are only partially informative at this level of relatedness. A comparison of mitochondrial cytochrome oxidase II (Janecek et al. 1996) or cytochrome *b* sequences (Hassanin and Douzery 1999a; Hassanin and Douzery 1999b; Schreiber *et al.* 1999) from incomplete species panels led to partial phylogenies. Janecek et al. (1996) found an anomalous association of yak with taurine cattle, which was explained by the sampling of an animal descending from a zebu via the maternal lineage (Ward *et al.* 1999). However, one consistent finding has been an unexpected divergence of the mitochondrial genomes from American and European bison (Janecek *et al.* 1996; Schreiber *et al.* 1999; Ward *et al.* 1999). Comparison of AFLP fingerprints of the nuclear genomes (Buntjer *et al.* 2002) suggested that reticulation has played a role during the evolution of the bovine species. Further, a microsatellite-based tree (Ritz *et al.* 2000) agreed only partially with the AFLP and mitochondrial trees.

In order to construct a phylogeny reflecting both the maternal and paternal lineages, we have compared three mitochondrial and two Y-chromosomal gene segments from the extant *Bos* and *Bison* species. Our mitochondrial DNA data indicate an early divergence of four maternal lineages in the *Bos* and *Bison* species and confirmed the anomalous position of the wisent. However, the Y-chromosomal phylogeny is entirely consistent with the obvious similarity and cross-fertility of the two bison species. This can be explained either by a combination of recent lineage sorting events or by a hybrid origin of wisent after male introgression.

2. Materials and methods

2.1 Samples

Blood or tissue was collected from the following species: ox (*Bos taurus*, Limousin breed), zebu (*Bos indicus*, Sahiwal breed, a gift from Dr. D.G. Bradley, Dublin), banteng (*Bos javanicus*, Blijdorp Zoo, Rotterdam), gayal (*Bos frontalis*, Mr. J.P.M. Janssen, Helvoirt, The

Table 2. Oligonucleotide primers for amplification of mitochondrial and Y-chromosomal DNA segments

DNA segment	Sequence (5→3')	Position
cytochrome *b*	TTCATCGACCTTCCAGCCCC	14564-14583
	TGAGTGTTAGTAGGTCTGCT	15518-15500
	ACAAATCCTCACAGGCCTATTC	14642-14663
	TAGGACGTATCCTATGAATGCT	14912-14891
cytochrome oxidase II	ATGGCATATCCCATACAACTAG	7374-7395
	ACTTTAGTGGGACTAACTCAAG	8004-8025
D-loop	AAAAATCCCAATAACTCAACACAG	15848-15871
	TACCATAGATGCTCCGGGTCAG	126-105
SRY	GTCTGCTGCACCTTCATC	701-714
	GTTCATGGGTCGCTTGAC	1243-1226
	TGCCAGGAGGTATTGAGG	891-908
	CAGAGGAGCAGTTATTTT	1903-1885
	CAATCATATGCTTCTTCTATGT	871-891
	GTACTTACTTATTGGGCT	1851-1834
	AAATTGAGATAAAGAGCGCCT	1798-1778
	GCCTGGACTTTCTTGTGCTTA	1723-1740
	CAGTGGGAACAAAAGACTAT	2347-2327
	CTACTTTAGCCTTATTTGC	2726-2709
ZFY	GAAACCCAATTAAAATATATGAAGCA	
	AGACCTGATTCCAGACAGTACCA	

For the mitochondrial primers the position numbers refer to Genbank entry NC_001567. Y-chromosomal primers are based on the *SRY* Genbank entry AB039748 and the *ZFY* gene (Cathey *et al.*, 1998)

Netherlands), gaur (*Bos gaurus*, Dr. D.G. Bradley, Dublin; Hellabrunn Zoo, Munich), yak *(Bos grunniens*, Artis Zoo, Amsterdam), bison *(Bison bison*, Artis), wisent (*Bison bonasus*, Artis), African buffalo (*Syncerus caffer*, Zimbabwe) and water buffalo *(Bubalus bubalis*, Potenza, Italy). Genomic DNA was extracted from blood using the guanidium-isothiocyanate method as described by Ciulla *et al.* (1988) or from tissues using the proteinase-K/SDS method described by Sambrook *et al.* (1989).

2.2 PCR amplification and sequencing

PCR was performed with 50 ng of genomic DNA, 50 ng primers specific for the mitochondrial and Y-chromosomal gene segments (see the supplementary table 2) in 25 µl standard PCR reaction buffer (1.5 mM $MgCl_2$, 0.2 mM dNTP's) and 1 U *Taq* polymerase. After 3 min at 95°C, 35 cycles were performed of 15 s at 95°C, 30 s 58°C and 45 s at 72°C, followed by a final extension step of 5 min at 72°C. Sequencing from both ends was performed with 200 ng PCR product, a Cy5 Big Dye terminator kit (Applied Biosystems) and an ABI Prism 310 Sequencer. Genbank accession codes are listed in the supplementary Table 3.

2.3 Tree reconstructions

Sequences were aligned with the program BioEdit
(Hall 2002, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the alignments were corrected manually. As indicated in the supplementary Fig. 3, large indels and variable regions where the alignment was ambiguous were not used for phylogeny reconstructions. Substitution saturation in the aligned sequences was measured with the program DAMBE (Xia *et al.* 2003). Partition homogeneity tests and maximum likelihood tree reconstructions with different realistic models (GTR+G, HKY85, HKY85 +G and F81+G, respectively) were performed as implemented in the PAUP packages (V4, Swofford 2000). Bayesian tree reconstructions were performed by using the program MrBayes (Huelsenbeck 2000). All trees were plotted with African buffalo and water buffalo as outgroups. Split decompositions in the mtDNA data (Bandelt and Dress 1992) were calculated and plotted using the Splitstree software (Huson 1998, http://bibiserv.techfak.uni-bielefeld.de/splits). A median

network plot of the Y chromosomal sequences (Bandelt *et al.* 1995) was constructed using the Network 3.1.0.1 program (Fluxus Technology Ltd, http://www.fluxus-engineering.com).

3. Results

3.1 Mitochondrial DNA

Segments of the cytochrome *b* gene (874 bp), the control region (522 bp without insertions) and cytochrome oxidase II gene (517 bp) from the bovine species were amplified and sequenced. In order to verify the origin of our samples, we compared our data with partial or overlapping sequences from several sources (see the supplementary Table 2). Generally, we found an intraspecies variation clearly lower than the interspecies variation and a clustering of sequences of the same species in trees constructed on the basis of all available data

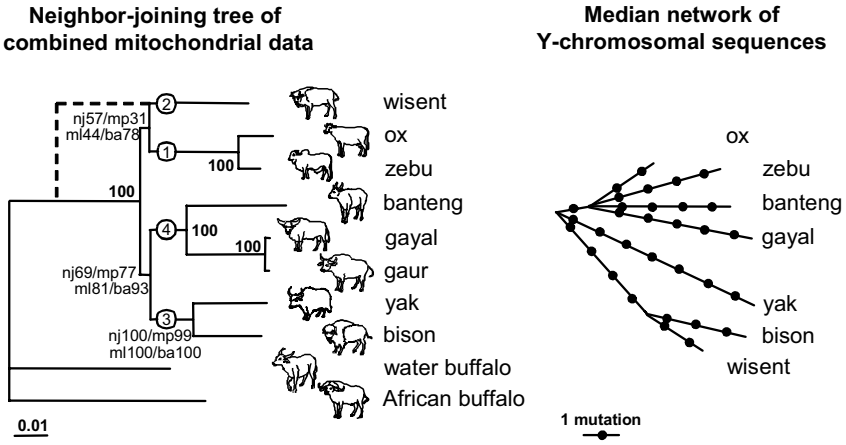

Figure 1. Phylogenetic trees of bovine species. In the neighbor-joining tree the circled numbers correspond to the numbering of lineages in the text. The figures near nodes indicate bootstrapping percentages of the neighbor-joining (nj) maximum parsimony (mp) and maximum likelihood with the GTR+G model (ml, Swofford 2000) or the fraction of times a given clade occurs in the trees sampled during Bayesian analysis (ba); the figures of 100 are generated by all four or three of the algorithms. The interrupted line indicates an alternative position of wisent, diverging from a cluster of lineages (1), (2) and (4).

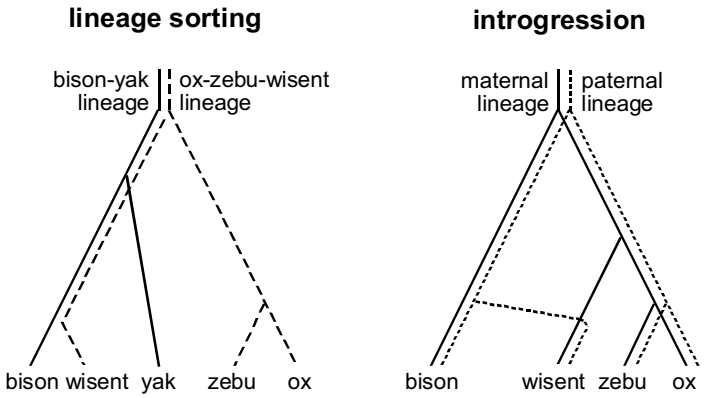

Figure 2. Explanations for the divergence of the mitochondrial DNA from bison and wisent.

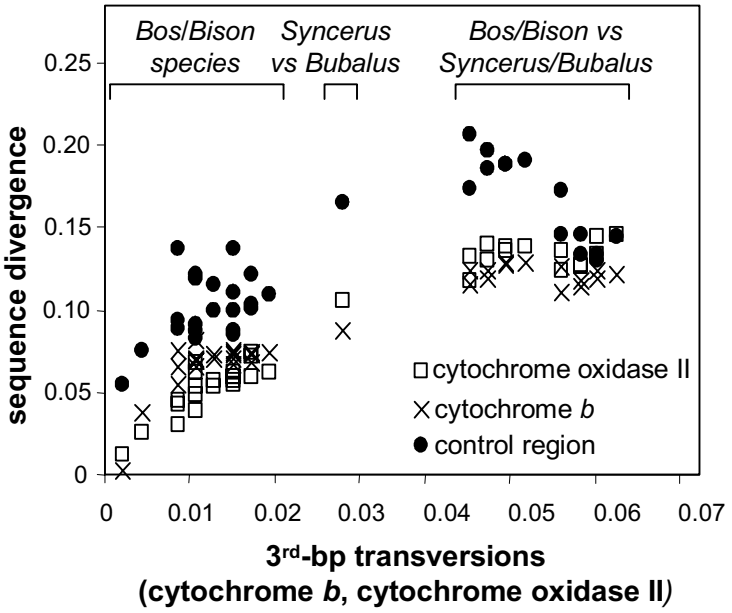

Figure. 4. Sequence divergence of cytochrome b, cytochrome oxidase II and the control region plotted agains the number of transversions in the cytochrome b and cytochrome oxidase genes, indicating saturation at more than 10 to 15 % sequence divergence.

Table 1. Clusters in mitochondrial trees: bootstrapping values and SplitsTree topologies.

Gene segment, algorithm	ox + zebu (1)	bison + yak (3)	banteng + gaur + gayal (4)	ox + zebu + wisent, (1)+(2)	(1) + (4)	(3) + (4) + (3)	(1) + (2) + (4)	(1) + (2) + (4)	(1) + (3)
cytochrome b									
Neighbor-joining	100	100	97	50	22	26	14	37	18
Maximum parsimony	99	87	86	23	45	19		46	39
SplitsTree	**3**	**1**	**1**						
cytochrome oxidase II									
Neighbor-joining	100	98	91	50	6	65	-	30	41
Maximum parsimony	100	97	79	39		74	-	20	53
Splitstree	**1**	**3**	**2**						
D-loop									
Neighbor-joining	99	46	99	82	-	20	49	9	-
Maximum parsimony	97	59	96	58	-	15	26	6	-
Splitstree	**2**	**3**	**3**						
Combined data									
Neighbor-joining	100	100	100	92	6	71	6	22	
Maximum parsimony	100	96	100	62		44	7	48	27
Maximum Likelihood	100	100	100	86	7	47	14	36	7
Bayesian analysis	100	100	100	90		68			
Splitstree	**1**	**1**	**1**	**1**		**1**			

(1), (2), (3) and (4) indicate the numbering of the mitochondrial lineages used in the text. The values for the Bayesian analysis correspond to the fractions the respective clades occur among the trees sampled. In the maximum-parsimony tree of the D-loop bison was clustered with lineages (1) and (2) and yak with lineage 4. The clusters generated by the SplitsTree algorithm have the following designations: **1**, common ancestor connected by one line to the rest of dendrogram (*i.e.*, complete bifurcation); **2**, connected by two lines; **3**, connected by three lines.

Table 3. Sequences from mitochondrial and Y-chromosomal DNA segments from bovine species.

Species	Mitochondrial DNA segment	Genbank accession codes
ox (*Bos taurus*)	cytochrome *b*	NC_001567, AF371961, D34635
	cytochrome oxidase II	NC_001567
	D-loop	NC_001567
	SRY	AB039748
	ZFY	**AY079140**, AF241271
zebu (*Bos indicus*)	cytochrome *b*	**AY079130**, [Y16064]
	cytochrome oxidase II	**AF348595**, [U18820]
	D-loop	AF162484[1], AF309097[1], AF3367381, L27714[1], U51806[1]
	SRY	**AY079145**, D10843
	ZFY	**AY079139**
banteng (*Bos javanicus*)	cytochrome *b*	**AY079131**, D82889, Y16058[1], D34636
	cytochrome oxidase II	U18821
	D-loop	AF162489[1]
	SRY	**AY079146**
	ZFY	**AY079139**
gaur *(Bos gaurus*)	cytochrome *b*	**AY079127**, Y16057
	cytochrome oxidase II	**AF348592**, U18818
	D-loop	**AF485067**, AF083371
	SRY	**AF495856**
	ZFY	**AY079135**
gayal (*Bos frontalis*)	cytochrome b	**AY079128**
	cytochrome oxidase II	**AF495858**
	D-loop	**AF485068**
	SRY	**AY079143**
	ZFY	**AY079136**

yak (*Bos grunniens*)	cytochrome b	**AY079129**, AF091631, Y16063
	cytochrome oxidase II	**AF348594** [U18819][2]
	D-loop	**AF485066**, AF083355
	SRY	**AY079144**, AF148463
	ZFY	**AY079137**
wisent (*Bison bonasus*)	cytochrome *b*	**AY079126**, Y15005, Y16061[1]
	cytochrome oxidase II	U62567[3]
	D-loop	**G9899097**, AF083356, U12953[1]
	SRY	**AY079142**, Z30321
	ZFY	**AY079142**
bison (*Bison bison*)	cytochrome *b*	**AY079125**, AF036273, Y16060
	cytochrome oxidase II	U62568
	D-loop	**G9899096**, AF83357[1]
	SRY	**AY079141**
	ZFY	**AY079133**
water buffalo	cytochrome *b*	**AY079132**, D88635, AF371962
(Bubalus bubalis)	cytochrome oxidase II	**AF495857**
	D-loop	AF197196[1], AF250508[1]
African buffalo	cytochrome *b*	AF036275, Y16055[1]
(*Syncerys caffer*)	cytochrome oxidase II	U18825[1]
	D-loop	AF028843[1]

Underlined sequences have been used for the tree reconstructions of figure1 and Table 2; the other sequences served to verify the true and pure species origin of the samples. Bold printing indicates sequences are from this study. Anomalous sequences are placed between square brackets.

1 First entry from a series of similar sequences from individuals of the same species

2 In phylogenetic trees closely associated with zebu sequences

3 Sequence identical to the sequence from one of our own samples.

(not shown). However, three previously published sequences were not consistent (Table 2). A cytochrome oxidase II sequence from one yak can be identified as descending from a zebu cow, a cytochrome oxidase II sequence from a Brahman zebu is clearly of taurine origin (Janecek *et al.* 1996; Ward *et al.* 1999) and a short cytochrome *b* segment from a dwarf zebu (Schreiber *et al.* 1999) is probably anomalous, since it deviates from all other bovine sequences. We conclude that our mitochondrial sequences represent the authentic maternal lineages of the respective bovine species.

Plots of the sequence divergence against the percentage transversions in the third codon positions of the cytochrome *b* and cytochrome oxidase II genes (Fig.4 of the supplementary material) showed that at more than 10 to 15 % sequence divergence the control region and, to a lesser degree, the cytochrome *b* gene started to become saturated with mutations. This has been observed before (Brant and Orti 2002; Rosel *et al.* 1995) and was most notable in the comparisons of the *Bos* and *Bison* species with the African and water buffalo, respectively. However, saturation analysis (Xia *et al.* 2003) indicated that the sequences of cytochrome *b*, cytochrome oxidase II as well as the control region were phylogenetically informative. A partition homogeneity test (Farris *et al.* 1994) further indicated that the tree topologies generated by the cytochrome *b*, cytochrome oxidase II and control region, respectively, were not significantly different (P = 0.25), although this value appeared to be sensitive to small modifications in the alignment of the control region.
Neighbor-joining, maximum parsimony, maximum likelihood as well as Bayesian analysis consistently grouped the mitochondrial sequences of the *Bos* and *Bison species* in four lineages: (1) ox and zebu; (2) wisent; (3) bison and yak; and (4) banteng, gayal and gaur (Fig.1). The clustering of these lineages depended on both the algorithm and the mitochondrial gene segment (Table 1). The cytochrome *b* and cytochrome oxidase II data favor an early split-off of the wisent. However, this not compatible with data of the control regions (Table 1), which indicates an association of wisent with lineage (1). The same cluster is also generated by Bayesian and SplitsTree analysis of the cytochrome *b* sequences and by the combined data. Most trees further indicated a clustering of lineages (3) and (4).

3.2 Y-chromosomal sequences

Previously sequenced *SRY* fragments from other banteng, yak and wisent bulls (see supplementary table 2) agreed with our data, indicating the authenticity of the paternal lineages of these animals. We identified 32 mutations in 1510 bp from *SRY* and 437 bp from *ZFY*, which are both on the male-specific part of the Y-chromosome. From gaur and water buffalo only partial SRY sequences were available. The gaur sequences were similar to the corresponding sequences from gayal (results not shown).

The partition homogeneity test showed that the Y-chromosomal and mitochondrial topologies are significantly different ($P = 2 \times 10^{-5}$). A median network (Fig. 1) shows which mutations are shared between species (Bandelt *et al.* 1995). At one position a G residue was shared by yak, American bison, wisent and water buffalo, suggesting a G $\rightarrow$A change in the *Bos* species. These species do not share other mutations, so taurine cattle, zebu, banteng and gayal all occupy separate branches in the network. However, an association of American bison and wisent is supported by four mutations.

4. Discussion

After diverging for 1 Myr or less, the speciation within the *Bovini* is not yet complete and female hybrid offspring from *Bos* and *Bison* species are fertile. AFLP fingerprints of nuclear DNA indicated a sharing of biallelic polymorphisms and reticulation (Buntjer *et al.* 2002).

By comparison of our data with published sequences the authentic maternal and paternal descendance of our samples has been verified. Except for gaur (*Bos gaurus*) and gayal (*Bos frontalis*) we found a clear segregation of species-specific mitochondrial sequences. This was confirmed by PCR-RFLP tests on mitochondrial DNA of several bovine individuals (Verkaar *et al.* 2003).

Trees constructed on the basis of the separate mitochondrial DNA segments were generally in agreement with the topology shown in Fig. 1, but alternative clusterings were supported by bootstrapping values of up to 65 % (Table 1). Although the mitochondrial gene segments generated different topologies, the partition homogeneity test suggested that these are stochastic effects of partitioning.

Phylogenetic tree reconstructions indicate four distinct mitochondrial lineages, one of these represented by the wisent. The association of ox (*Bos taurus*) and zebu (*Bos indicus*) reflects the fertility of female as well male hybrid offspring and their divergence time of 100.000 to 200.000 years (Bradley *et al.* 1996). The clustering of the South-East Asian *Bos* species banteng and gaur is in agreement with previous data (Janecek *et al.* 1996; Schreiber *et al.* 1999) and is also consistent with the former designation of these species as *Bibos javanicus* and *Bibos gaurus*, respectively. Our data also indicate a close relatedness of the wild gaur and the domestic gayal or mithan (*Bos or Bibos frontalis*) and do not support alternative hypotheses on the origin of the gayal (Simoons 1984).

We further provide molecular evidence for a relation of bison (*Bison bison*) and yak (*Bos mutus*), which confirms morphological evidence (Geraads 1992; Groves 1981), their association in trees of incomplete sets of *Bovini* species (Hassanin and Douzery 1999a; Schreiber *et al.* 1999; Ward *et al.* 1999) and AFLP fingerprinting (Buntjer *et al.* 2002).
An early association of the banteng-gaur-gayal and the bison-yak lineages (Fig. 1) has the highest bootstrapping values and appears on the basis of the present data the most likely topology. This would imply that *Bos* is not a monophyletic taxon.

The most striking feature of the bovine phylogeny is the divergence of the mitochondrial lineages of bison (*Bison bison*) and wisent (*Bison bonasus*), species that can produce completely fertile hybrid offspring and have similar AFLP patterns (Buntjer *et al.* 2002) as well as Y-chromosomal sequences (this study). The present data on three mitochondrial DNA segments from a complete panel of bovine species indicate either an association of wisent with the ox-zebu lineage or, with about equal bootstrapping support, an earlier divergence of wisent. However, since the latter possibility is not supported at all by the control region data and is also not indicated by Bayesian and SplitsTree analysis, we propose the wisent-zebu-indicus clustering as the most plausible topology.

Three explanations have been proposed for the mitochondrial divergence of the bison species (Janecek *et al.* 1996): phenotypic convergence, lineage sorting and an ancient hybridization event. The first explanation can be ruled out on the basis of the complete interfertility of bison and wisent and the similarity of their AFLP patterns and Y-chromosomal sequences. Lineage sorting is an obvious possibility and would imply an early divergence of the (American) bison/yak and zebu/taurine/wisent mitochondrial sequences, fol-

lowed by a split of the zebu/taurine and wisent lineages before the first split on the species level (Fig. 2). In the branch leading to both bison species, two lineages would have persisted. By four sorting events, a single lineage was fixed in the branches leading to zebu/ox, yak, American bison and European bison, respectively, possibly during a recent or former population bottleneck.

Ancient hybridization would have been facilitated by the social structure of herd species, in which most males are excluded from reproduction by the dominating bulls and live solitarily or form separate herds. Interestingly, an introgression of bison bulls in wisent herds (Fig. 2) would be compatible with paleontological records (Flerov 1979; Kurtén 1968; McDonald 1980; Pucek 1986; Pucek 1991; Skinner and Kaisen 1947). These data indicate that during the late Pliocene-early Pleistocene the ancestral bison originated in Southern Asia from the ancestral *Leptobos* and spread over the temperate zone. In the early Pleistocene immigrants in North America were the ancestors of the *Bison antiquus*, from which the extant *Bison bison* descended (McDonald 1980). European variants were the *Bison priscus* (steppe wisent) and *Bison schoetensacki* (Pleistocene woodland wisent). *Bison priscus* has been depicted in cave paintings of Altamira and Lascaux 17.000 to 19.000 years ago, but died out about 10.000 yr BC (Harington 1996; Kurtén 1968). Like the extant wisent (*Bison bonasus*) it had relatively long hind legs, but it was larger in size and is not considered to be its ancestor. A recent mitochondrial DNA analysis of *Bison priscus* bones (Nielsen-Marsh *et al.* 2002) revealed a control region sequence that was more related to *Bison bison* (4.5% difference, see the supplementary Fig. 5) than to *Bison bonasus* (7.2%). Although the much rarer *Bison schoetensacki* was of the same size as *Bison bonasus*, it is also considered to have died out without extant descendants (Kurtén 1968). *Bison bonasus* appeared not before the late Pleistocene or Holocene (Flerov 1979; Pucek 1986) and is supposed to descend from American bisons (Kurtén 1968; McDonald 1980). Combining these paleontological data with male introgression as explanation of the molecular data, we propose a tentative scenario in which the phenotype as well as the nuclear DNA of an ancient Eurasian cattle-like population was changed by systematic introgression of *Bison bison, Bison priscus* or possibly *Bison schoetensacki* bulls. Repeated introgression eventually created a new species with a bison-like appearance, autosomal genes and Y-chromosome, but with the original mitochondrial DNA from the maternal ancestor. This

scenario would explain the sudden paleontological appearance of the extant wisent without clearly identifiable ancestors and may be amenable to experimental testing by molecular analysis of fossil remains (Nielsen-Marsh *et al.* 2002; Willerslev *et al.* 2003).

There are three canonical categories of speciation, allopatry by differentiation after genetic isolation, parapatry by differentiation of neighboring populations, often separated by a hybrid transition zone (Hewitt 2001), and sympatry by differentiation within the same region. Peripatry (Barraclough and Vogler 2000) after isolation of a small founder population is a special case of allopatry. However, these modes of speciation do not include a relatively fast conversion of an existing population by male introgression. Here, we propose the term *transpatry*, which would not be common but rather a phenomenon unique for species with a herd organization, female philopatry and few dominating males. Although the wisent transpatry is still hypothetical, it probably has played a role in the evolution of deer (Cathey *et al.* 1998), macaques (Tosi *et al.* 2000), the domestic alpaca (Kadwell *et al.* 2001) and goat species (N. Pidancier, G. Luikart, P.J. Weinberg and P. Taberlet, unpublished results). The breeding of African zebus by crossing imported Indian bulls into taurine breeds (Bradley *et al.* 1996) may be considered as an artificial form of transpatry, which has expanded the domestic habitat range of the endogenous cattle.

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References

Bandelt, H. J., and A. W. Dress, 1992 Split decomposition: a new and useful approach to phylogenetic analysis of distance data. Mol Phylogenet Evol **1:** 242-252.

Bandelt, H. J., P. Forster, B. C. Sykes and M. B. Richards, 1995 Mitochondrial portraits of human populations using median networks. Genetics **141:** 743-753.

Barraclough, T. G., and A. P. Vogler, 2000 Detecting the Geographical Pattern of Speciation from Species-Level Phylogenies. Am Nat **155:** 419-434.

Bohlken, H., 1961 Haustiere und Zoologische Systematik. Z. Tier. Zuchtungsbiol. **76:** 107-113.

Bradley, D. G., D. E. MacHugh, P. Cunningham and R. T. Loftus, 1996 Mitochondrial diversity and the origins of African and European cattle. Proc Natl Acad Sci U S A **93:** 5131-5135.

Brant, S. V., and G. Orti, 2002 Molecular phylogeny of short-tailed shrews, Blarina (Insectivora: Soricidae). Mol Phylogenet Evol **22:** 163-173.

Buntjer, J. B., M. Otsen, I. J. Nijman, M. T. Kuiper and J. A. Lenstra, 2002 Phylogeny of bovine species based on AFLP fingerprinting. Heredity **88:** 46-51.

Cathey, A. C., J. W. Bickham and J. C. Patto, 1998 Introgressive hybridization and noncongruent evolutionary history of maternal and paternal lineages in north-american deer. Evolution **52:** 1224-1229.

Chikuni, K., Y. Mori, T. Tabata, M. Saito, M. Monma *et al.*, 1995 Molecular phylogeny based on the kappa-casein and cytochrome b sequences in the mammalian suborder Ruminantia. J Mol Evol **41:** 859-866.

Ciulla, T. A., R. M. Sklar and S. L. Hauser, 1988 A simple method for DNA purification from peripheral blood. Anal Biochem **174:** 485-488.

Farris, J. S., M. Källersjö, A. G. Kluge and C. Bult, 1994 Testing significance of incongruence. Cladistics **10:** 315-319.

Flerov, K. K., 1979 Systematics and evolution. Pp 9-127 in E.V. Sokolov ed. European bison. Morphology , systematics, evolution, ecology. Nauka Pibl., Moscow (in Russian).

Geraads, D., 1992 Phylogenetic analysis of the tribe Bovini (Mammalia: Artiodactyla). Zool. J. Linn. Soc. **19:** 264-278.

Groves, C. P., 1981 Systematic relationships in the Bovini (Artiodactyla, Bovidae). Z. Zool. Syst. Evol. **19:** 264-278.

Hall, T., 2002 Bioedit. Biological sequence alignment editor for Windows 95/97/NT (version 5/0/9). North Carolina State University.

Harington, 1996 Yukon Beringia interpretive centre. http://www.beringia.com.

Hassanin, A., and E. J. Douzery, 1999a Evolutionary affinities of the enigmatic saola (Pseudoryx nghetinhensis) in the context of the molecular phylogeny of Bovidae. Proc R Soc Lond B Biol Sci **266:** 893-900.

Hassanin, A., and E. J. Douzery, 1999b The tribal radiation of the family Bovidae (Artiodactyla) and the evolution of the mitochondrial cytochrome b gene. Mol Phylogenet Evol **13:** 227-243.

Hewitt, G. M., 2001 Speciation, hybrid zones and phylogeography - or seeing genes in space and time. Mol Ecol **10:** 537-549.

Huelsenbeck, J. P., 2000 MrBayes: Bayesian inference of phylogeny (version 2.01). Distrubuted by the author Dept. Biology, Univ. Rochester.

Huson, D. H., 1998 SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics **14:** 68-73.

Janecek, L. L., R. L. Honeycutt, R. M. Adkins and S. K. Davis, 1996 Mitochondrial gene sequences and the molecular systematics of the artiodactyl subfamily bovinae. Mol Phylogenet Evol **6:** 107-119.

Kadwell, M., M. Fernandez, H. F. Stanley, R. Baldi, J. C. Wheeler *et al.*, 2001 Genetic analysis reveals the wild ancestors of the llama and the alpaca. Proc R Soc Lond B Biol Sci **268:** 2575-2584.

Kurtén, B., 1968 Pleistocene mammals of Europe. Weidenfeld and Nicolson, Londen.

Lenstra, J. A., and D. G. Bradley, 1999 Systematics and phylogeny of cattle. CAB Intl. Ed. by R. Fries and A. Ruvenski**:** 1-14.

McDonald, J. N., 1980 North American Bison. Their classification and evolution. University of California Press, Berkeley.

Nielsen-Marsh, C. M., P. H. Ostrom, H. Gadhi, B. Shapiro, A. Cooper *et al.*, 2002 Sequence preservation of osteocalcin and mitochondrial DNA in bison bones older than 55 ka. Geology **30:** 1099-1102.

Nijman, I. J., D. G. Bradley, O. Hanotte, M. Otsen and J. A. Lenstra, 1999 Satellite DNA polymorphisms and AFLP correlate with Bos indicus-taurus hybridization. Anim Genet **30:** 265-273.

Pucek, Z., 1986 Bison bonasus (Linnaeus, 1758)-Wisent. Pp. 278-315 in Handbuch der Säugetier Europas, Aula Verlag, GmbH, Wiesbaden.

Pucek, Z., 1991 History of the European bison and problems of its protection and management. Pp.19-39 in B. Bobek, K., Perfanoski and W. Regelin, eds. Trans. 18th. UUGB Congress, Krakow, 1987, Swiat Press, Krakow-Warzawa.

Ritz, L. R., M. L. Glowatzki-Mullis, D. E. MacHugh and C. Gaillard, 2000 Phylogenetic analysis of the tribe Bovini using microsatellites. Anim Genet **31:** 178-185.

Rosel, P. E., M. G. Haygood and W. F. Perrin, 1995 Phylogenetic relationships among the true porpoises (Cetacea:Phocoenidae). Mol Phylogenet Evol **4:** 463-474.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 Molecular cloning. A laboratory manual. Cold spring harbor laboratory press, Cold spring harbor, USA.

Schreiber, A., I. Seibold, G. Notzold and M. Wink, 1999 Cytochrome b gene haplotypes characterize chromosomal lineages of anoa, the Sulawesi dwarf buffalo (Bovidae: Bubalus sp.). J Hered **90:** 165-176.

Simoons, F. J., 1984 Gayal or Mithan. Pp. 34-38 in I.L. MASON, ed. Evolution of domesticated animals. Longman, London and New York.

Skinner, M. F., and O. C. Kaisen, 1947 The fossil bison of Alaska and preliminary revision of the genus Bison. Bull. Amer. Mus. Nat. Hist. **89:** 123-156.

Swofford, D. L., 2000 PAUP: phylogenetic analysis using parsimony and other methods (version 4.0). Sinauer Associates, Sunderland MA.

Tosi, A. J., J. C. Morales and D. J. Melnick, 2000 Comparison of Y Chromosome and mtDNA Phylogenies Leads to Unique Inferences of Macaque Evolutionary History. Mol Phylogenet Evol **17:** 133-144.

Verkaar, E. L. C., H. Vervaecke, C. Roden, L. Romero Mendoza, M. W. Barwegen *et al.*, 2003 Paternally inherited markers in bovine hybrid populations. submitted.

Ward, T. J., J. P. Bielawski, S. K. Davis, J. W. Templeton and J. N. Derr, 1999 Identifiction of domestic cattle hybrids in wild cattle and bison species: a general approach using mtDNA markers and the parametric bootstrap. Animal Conservation**:** 51-57.

Willerslev, E., A. J. Hansen, J. Binladen, T. B. Brand, M. T. Gilbert *et al.*, 2003 Diverse plant and animal genetic records from Holocene and Pleistocene sediments. Science **300:** 791-795.

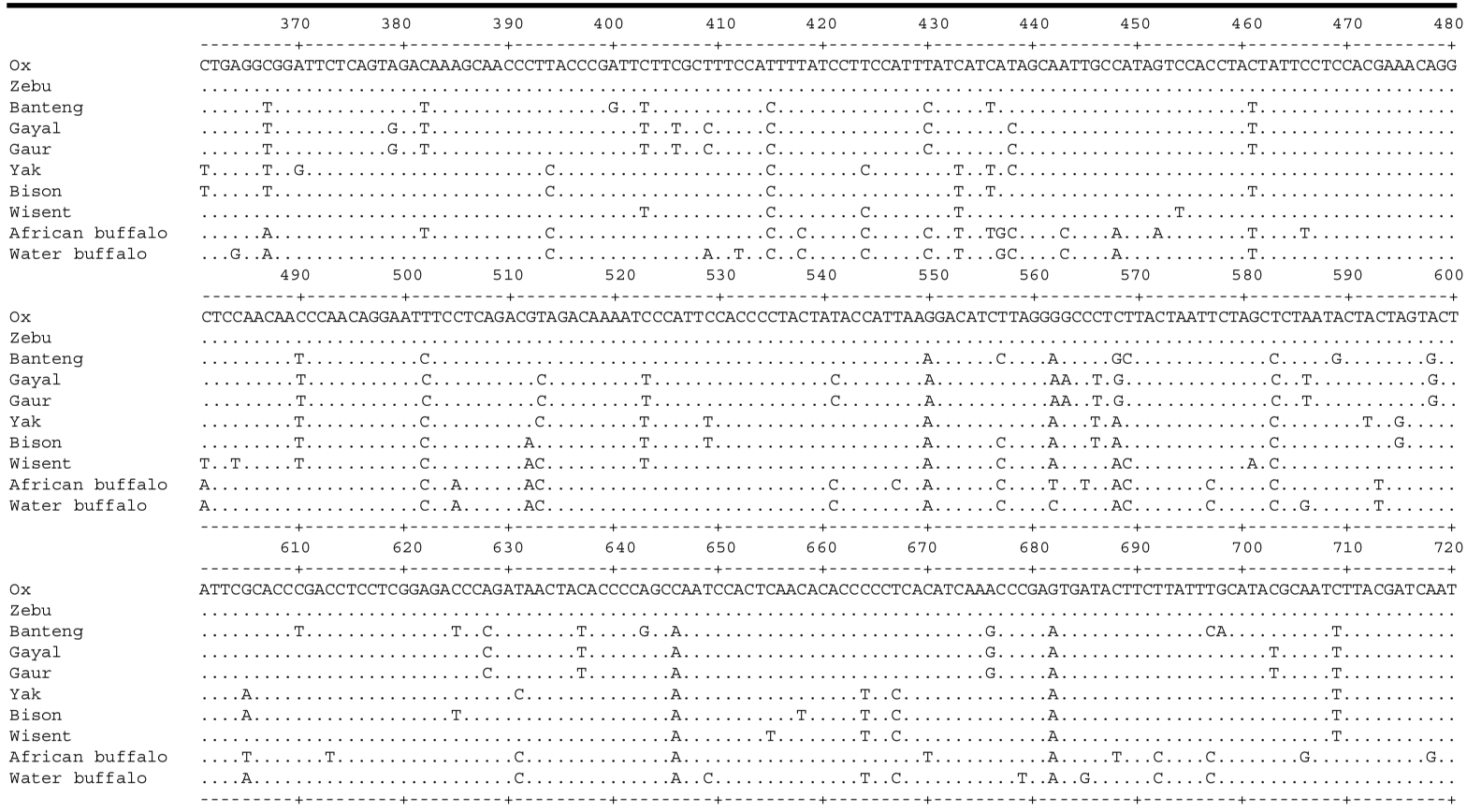
Xia, X., Z. Xie, M. Salemi, L. Chen and Y. Wang, 2003 An index of substitution saturation and its application. Mol Phylogenet Evol **26:** 1-7.

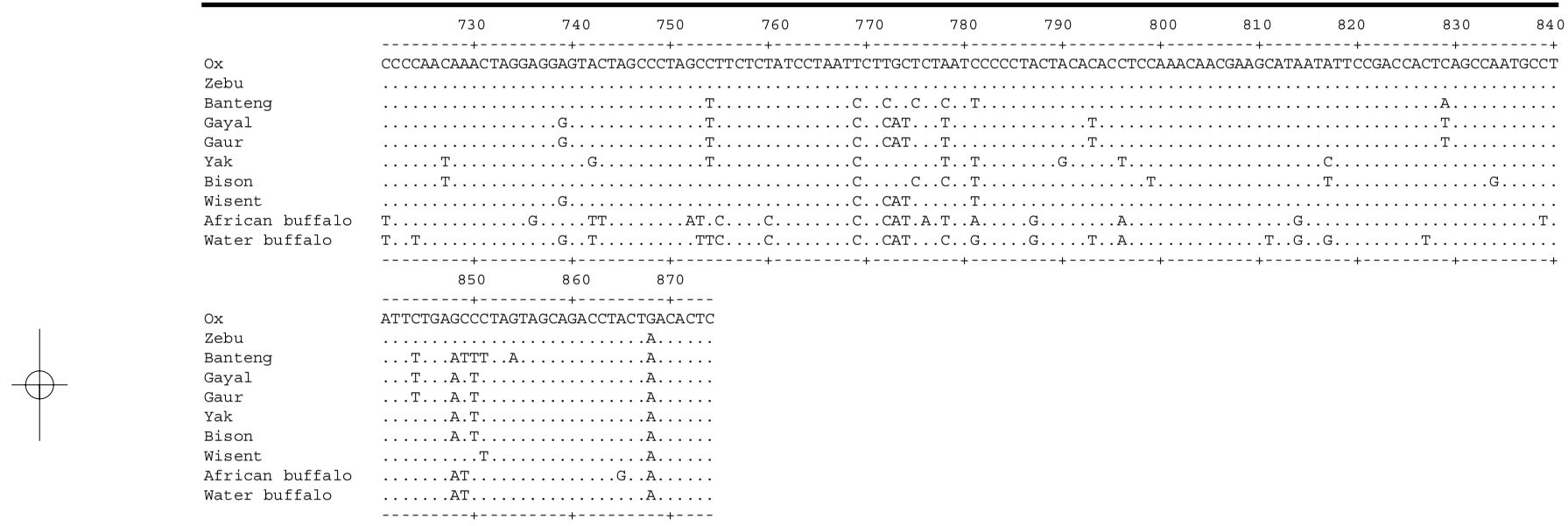
Supplement Chapter 3

Figure 3. - Sequence alignments. Lower case letters in the alignment of the control region indicate sequence positions not considered during the tree reconstructions.

Cytochrome *b*

```
                  10        20        30        40        50        60        70        80        90        100       110       120
                  ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
Ox                AATCCTCACAGGCCTATTCCTAGCAATACACTACACATCCGACACAACAACAGCATTCTCCTCTGTTACCCATATCTGCCGAGACGTGAACTACGGCTGAATCATCCGATACATACACGC
Zebu              ............................................................................................................................
Banteng           ............................T......C.....................C.....T.............................T..T.............
Gayal             ...........................................T.....................C.....................A.............T.................
Gaur              ...........................................T.....................C.....................A.............T.................
Yak               ...........................................T.....................C...G.............................T........T........
Bison             T...........................................T..............T.....C...G.............................................
Wisent            ........................G.....................................C..C..T.................A.............T.................
African buffalo   ............T.............T........A..T.................T..C..CG....C......N.......T........A.....T..T........G.....
Water buffalo     ...........................................................C..CG....C.....................T..A.....T..T.............
                  ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                  130       140       150       160       170       180       190       200       210       220       230       240
                  ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
Ox                AAACGGAGCTTCAATGTTTTTTATCTGCTTATATATGCACGTAGGACGAGGCTTATATTACGGGTCTTACACTTTTCTAGAAACATGAAATATTGGAGTAATCCTTCTGCTCACAGTAAT
Zebu              ............................................................................................................................
Banteng           ........................T.....G.....................C....C.............C..C.............C.................A..T........
Gayal             ........................T.....................................C..C.............C.................A..T........
Gaur              ........................T.....................C.....................C..C.............C.................A..T........
Yak               ................A..C.................A..........G...C.....................C..C.........................T.....A..T........
Bison             ................A..C.....................C..........C.................T..C..C.....................................A..T........
Wisent            T.....................C.....................C.......T.....................C...........T..C..A..T..G.....
African buffalo   ................A..C..C.............A.....................C....C..T..A..C.........T.............C..C...........C..AT.........
Water buffalo     ................A.....C.............A.................A....C.....A..A..T..C.................C..C.........T..AT.AT..G.......
                  ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                  250       260       270       280       290       300       310       320       330       340       350       360
                  ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
Ox                AGCCACAGCATTTATAGGATACGTCCTACCATGAGGACAAATATCATTCTGAGGAGCAACAGTCATCACCAACCTCTTATCAGCAATCCCATACATCGGCACAAATTTAGTCGAATGAAT
Zebu              ............................................................................................................................
Banteng           ............C........T..A.....................................T..T.........C...........................................
Gayal             ...T........C.....G..T..A...........G.....G.....T.........................C.............T...........................
Gaur              ...T........C.....G..T..A...........G.....G.....T.........................C.............T...........................
Yak               ...T........C.............A.....................T.....G...........T.........C...........T.....................G..
Bison             ............C.............A.....................T.....G...........T.........C.....................C.............
Wisent            ...T........C...........GT.....................T.................T.........C.....................C.............
African buffalo   ...T..G.....C........T..A..G.....................G........A..T..T..T...C.C.............T...........CC.............
Water buffalo     .....................A..G.................T.........G.....................TC.C.................T..T..T.G.C.G..T..G.....
                  ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
```



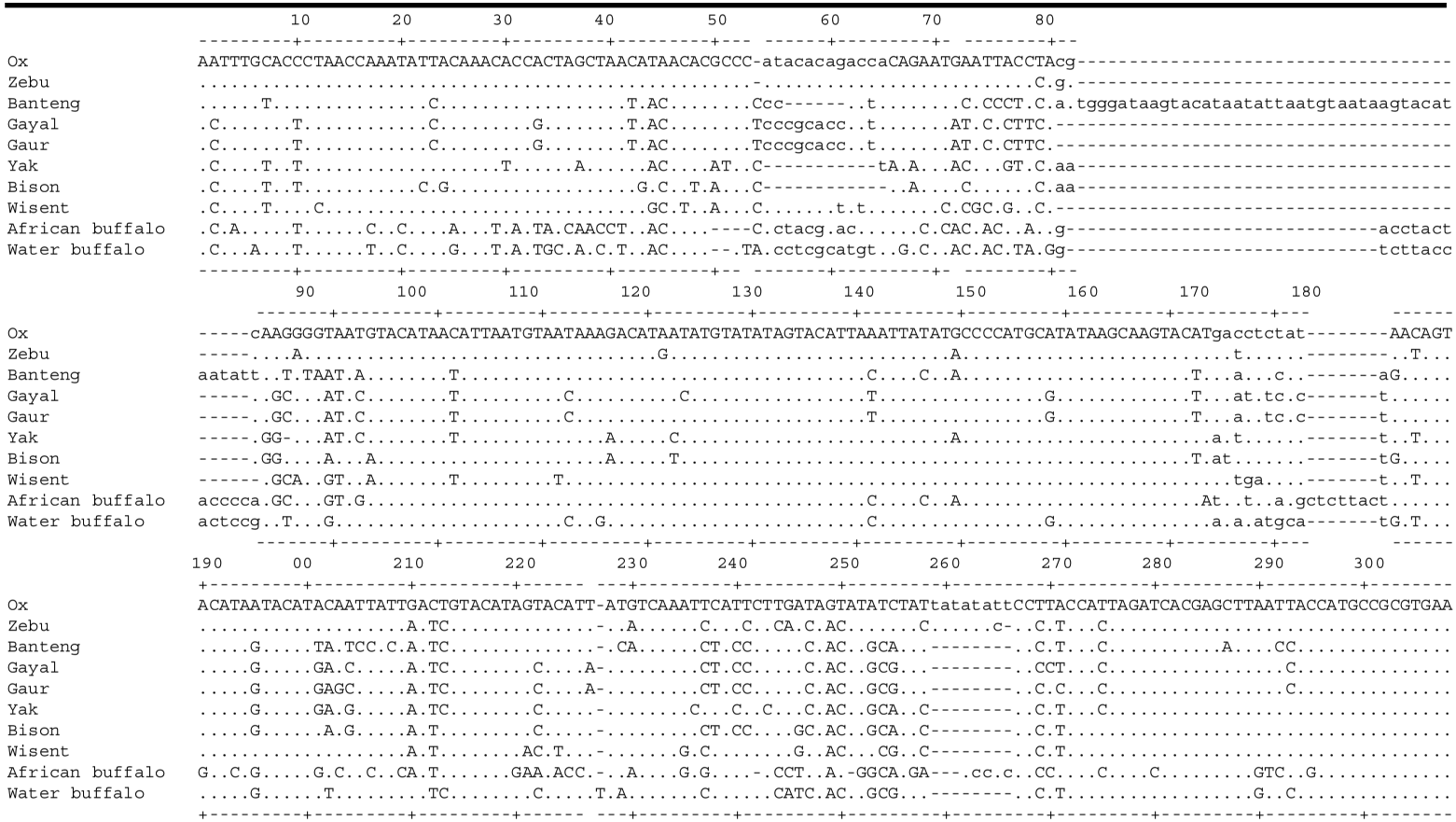


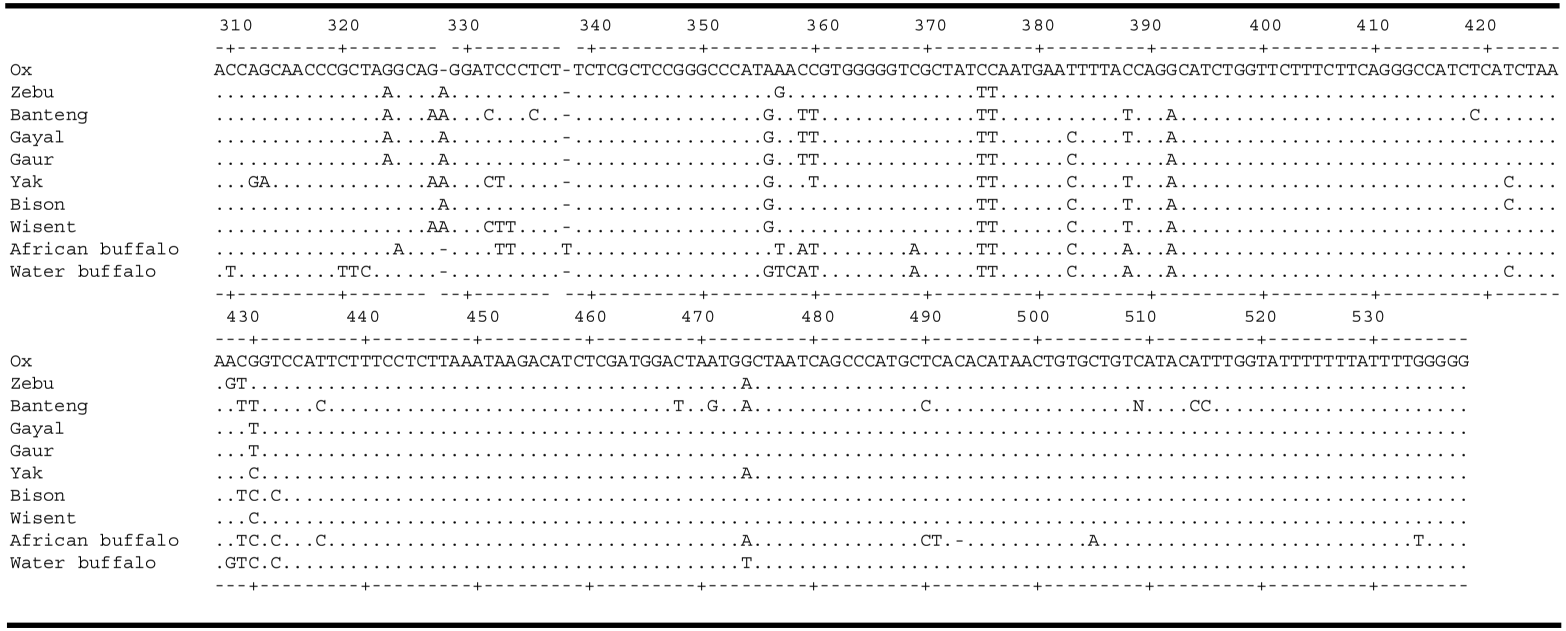
Cytochrome oxidase II

```
                 10        20        30        40        50        60        70        80        90        100       110       120
                 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
Ox               GCTAATAATTGTCTTCTTAATTAGCTCATTAGTACTTTACATTATTTCACTAATACTAACGACAAAGCTGACCCATACAAGCACGATAGATCCACAAGAAGTAGAGACAATCTGAACCAT
Zebu             ..........................................................A.............................G.............................
Banteng          ..........................................C........G..............A.....T...........A......G.............................
Gayal            A..........A.....................................................A.....T...........A......G.............................
Gaur             A..........A.....................................................A.....T...........A......G.............................
Yak              ..........................................C.........T....A.....A..A..T...........A......G...................T.........
Bison            ..........................................C..............A.....A.....T...........A......G.............................
Wisent           A.....................................................A...........T.....G.....A......G.........................T..
African buffalo  A........C..T........C...........G.....T..C..............G..A.....AT.A..T..C..C..T..A......G.............A.....T.....T..
Water buffalo    .........C.....T.................C.....T..............T....A.....A..A..T..C..C..T..A......G.............................
                 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                         130       140       150       160       170       180       190       200       210       220       230       240
                 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
Ox               TCTGCCCGCCATCATCTTAATTCTAATTGCTCTTCCTTCTTTACGAATTCTATACATAATAGATGAAATCAATAACCCATCTCTTACAGTAAAAACCATAGGACATCAGTGATACTGAAG
Zebu             ..........................................................................................................................
Banteng          .........T..T..T..............................T.........G..............T.....C.....G.............................
Gayal            .........T.....................C..................C.........................T.....C.....G.........T.....................
Gaur             .........T.....................C..................C.........................T.....C.....G.........T.....................
Yak              .T.......T..T..T.....................................................T...........C.....G......G.A.....................
Bison            CT.......T..T..T.....................................G...................TC.....G......G.A.....................
Wisent           .........T..T..............................G...T....T.....G...................C...........G..............A...........
African buffalo  ...A.....T..T...C.....T..............A..CC.............T..............T..C.........C..C..............G.........A...........
Water buffalo    ...A.....T..T...C.G...T.......A..C..G..CC.............T.....G...........C.........C..C..............G.....C..A...........
                 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                         250       260       270       280       290       300       310       320       330       340       350       360
                 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
Ox               CTATGAGTATACAGATTATGAGGACTTAAGCTTGGACTCCTACATAATTCCAACATCAGAATTAAAGCCAGGGGAGCTACGACTATTAGAAGTCGATAATCGAGTTGTACTACCAATAGA
Zebu             ..................................C.........................................................................................
Banteng          ...C..A..C.......................C.........................................................................G.........
Gayal            ...C..A..C.......................C.....................................................................GT.G.........
Gaur             ...C..A..C.......................C.....................................................................GT.G.........
Yak              ...C..A.........................C.....................................AT.................................G.........
Bison            ......A.........................C.....................................AT.................................G.........
Wisent           ......A..C......................C..........................................................................................
African buffalo  ......A........C.....A...C.G.....C............G..............G.........A.........T.G..............C..............C.....
Water buffalo    ......A..............A...C.G.....C.........................GC..........A..A.........C...................C...T....C..G..
                 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
```

```
                  370        380        390        400        410        420        430        440        450        460        470        480
                  ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
Ox                AATAACAATCCGAATGTTAGTCTCCTCTGAAGACGTATTACACTCATGAGCTGTGCCCTCTCTAGGACTAAAAACAGACGCAATCCCAGGCCGTCTAAACCAAACAACCCTTATATCGTC
Zebu              ................C.....................C.....................................T.....................................
Banteng           ................C.....................C.............C.....T...........G.........................G.....................A.
Gayal             ................C.............G......C.............C.....T...........G..............T...........G.....................A.
Gaur              ................C.............G......C.............C.....T...........G..............T...........G.....................A.
Yak               ..............AC.................T...C.............C..A..T...........G.........T.................G.....................A.
Bison             ..............AC.................T...C.............C.....T.....G.....G.........T.................G.....................A.
Wisent            ................C.....................C.............C..A.....................................................AA.
African buffalo   ..............A.........T.....G.....................C..A..T..C.....G.......................T.....G........T.....C..G..AA.
Water buffalo     G..G...........AC.G........C........TC..........G..C..A.....C..G..GT..........T........G..T.....G..............A..G..TA.
                  ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                          490        500        510
                  ---------+---------+---------+-------
Ox                CCGTCCAGGCTTATATTACGGTCAATGCTCAGAAATT
Zebu              ....................................
Banteng           ..........C....C..T...................
Gayal             ..........C....C..T...................
Gaur              ..........C....C..T...................
Yak               ..........C.G.......................C
Bison             ..........C....C..T.................C
Wisent            ..........C....C.....................
African buffalo   ...A.....TC.......T..C...........G..C
Water buffalo     ...A......C.G.....T.................C
                  ---------+---------+---------+-------
```

Control region





SRY

```
                10        20        30        40        50        60        70        80        90       100       110       120
                +---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
ox              ACACTTTTTGTATTTTTAAGCAGGTGTTAGCACATTCACAAATTCTGATTAGATGTAAACAAAGAAGAAAGCAGAGCCTTAATATCCTGTTAAGTAGCTTTGCTTGAGAAAGAGTAGGTT
zebu            ................................................................................................................
banteng         ................................................................................................................
gayal           ................................................................................................................
gaur            ................................................................................................................
yak             ................................................................................................................
wisent          ................................................................................................................
bison           ................................................................................................................
water buffalo                   ..........................................................C.....................................
                +---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                       130       140       150       160       170       180       190       200       210       220       230       240
                +---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
ox              GATGGGTTTGGGCTGACTGCCAGGACGTATTGAGGGGAGGTATTGGGGGCGGAGAAATAAATATTTCACTGTATATATTGCACTAAGTCAGTCTGTGGTAAGAACAACTTATGAATAGCA
zebu            ................................................................................................................
banteng         ................................................................................................................
gayal           ......................G.........................................................................................C....
gaur            ......................G.........................................................................................C....
yak             .G..............................................................G.................................................
wisent          .G..............................................................................................................
bison           .G..............................................................................................................
water buffalo   .G.....................................................................................A.......C.....................C
                +---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                       250       260       270       280       290       300       310       320       330       340       350       360
                +---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
ox              CCATAATTTTTAGAACGCTTACACCGCATATTACTTCCTCCCCTTTTAAACAGTGCAGTCGTATGCTTCTGCTATGTTCAGAGTATTGAACGACGATGTTTACAGTCCAGCTGTGGTACA
zebu            ................................................................................................................
banteng         ................................................................................................................
gayal           ................................................................................................................
gaur            ................................................................................................................
yak             ................T...............................................................................................
wisent          ...............................................................C................................................
bison           ...............................................................C................................................
water buffalo   ..G.....................................T.....T..................................................G.........
                +---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
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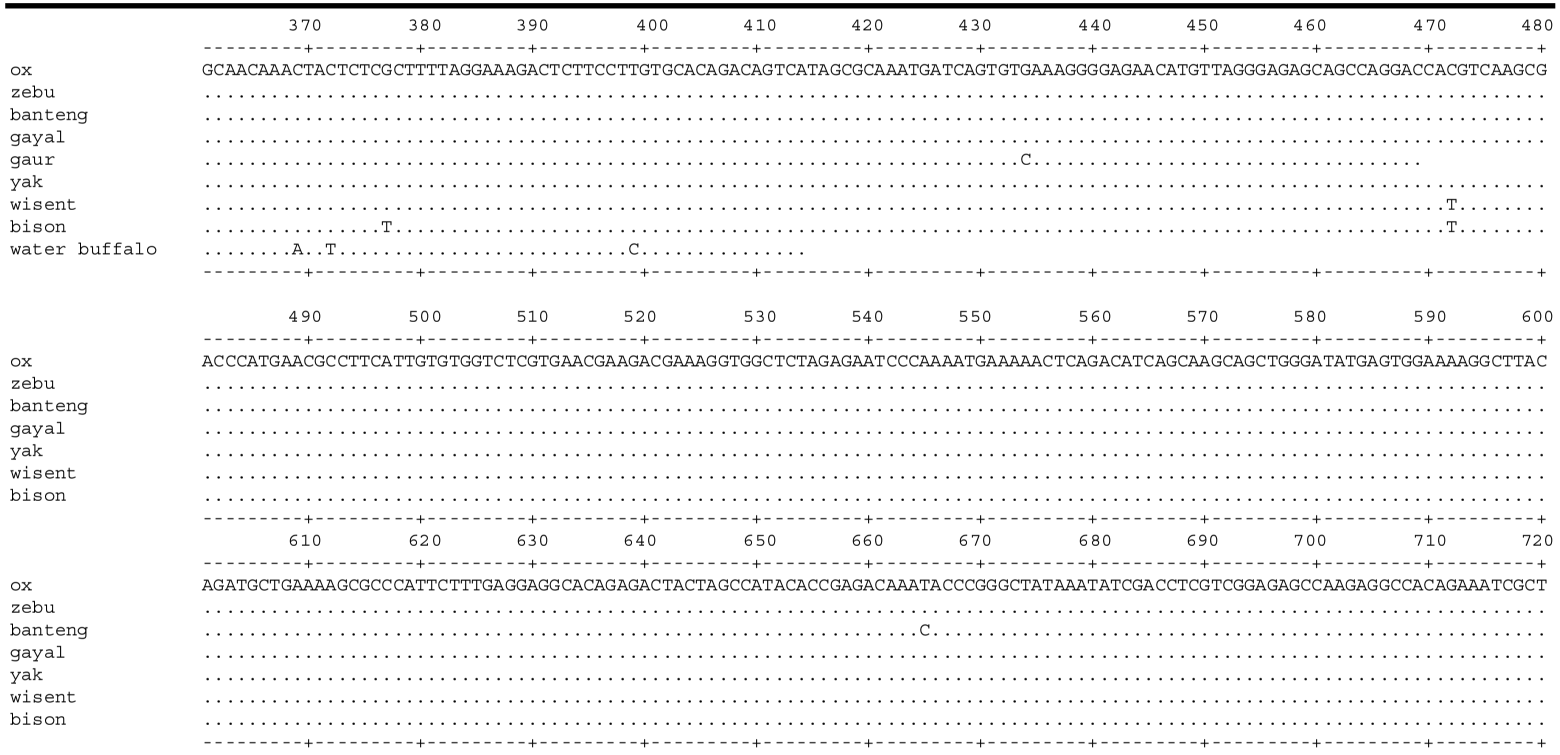
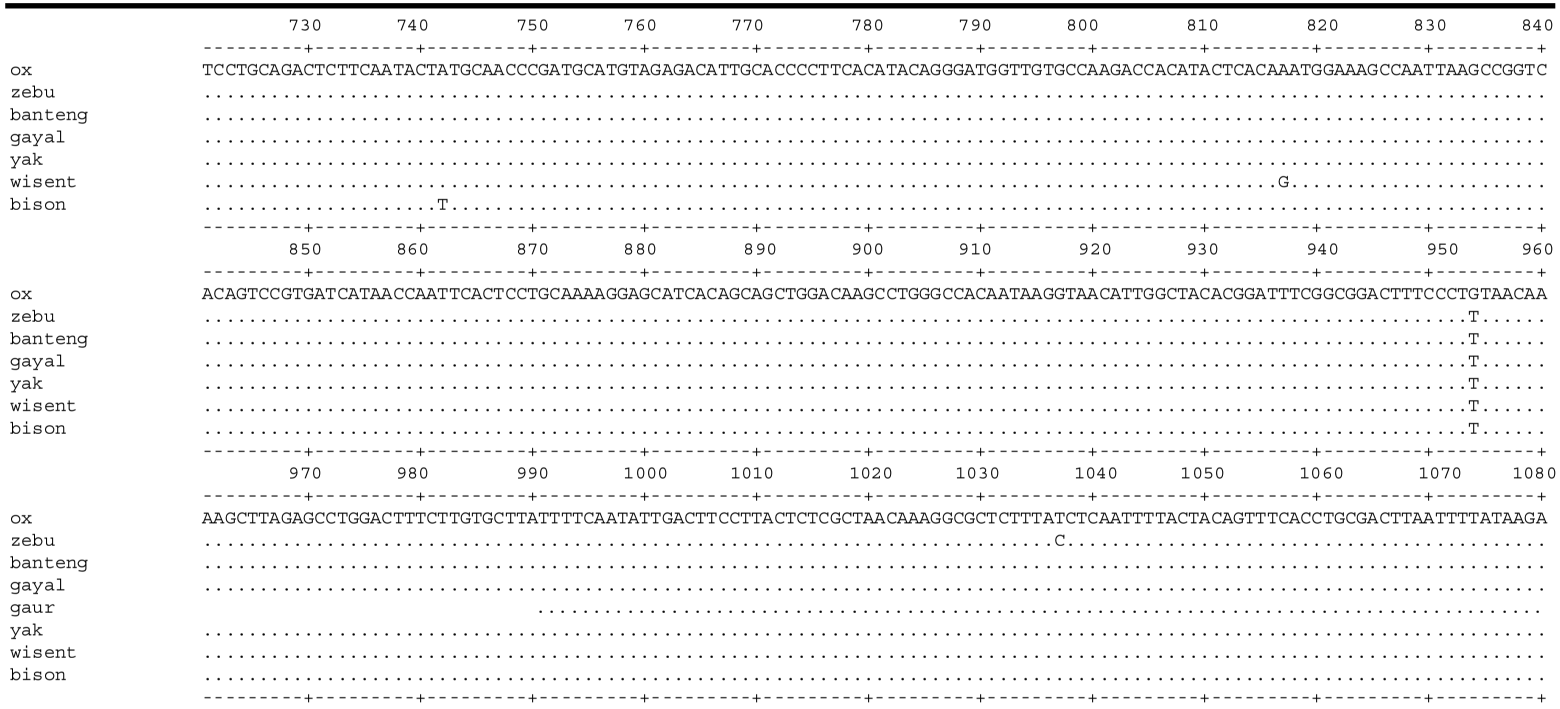
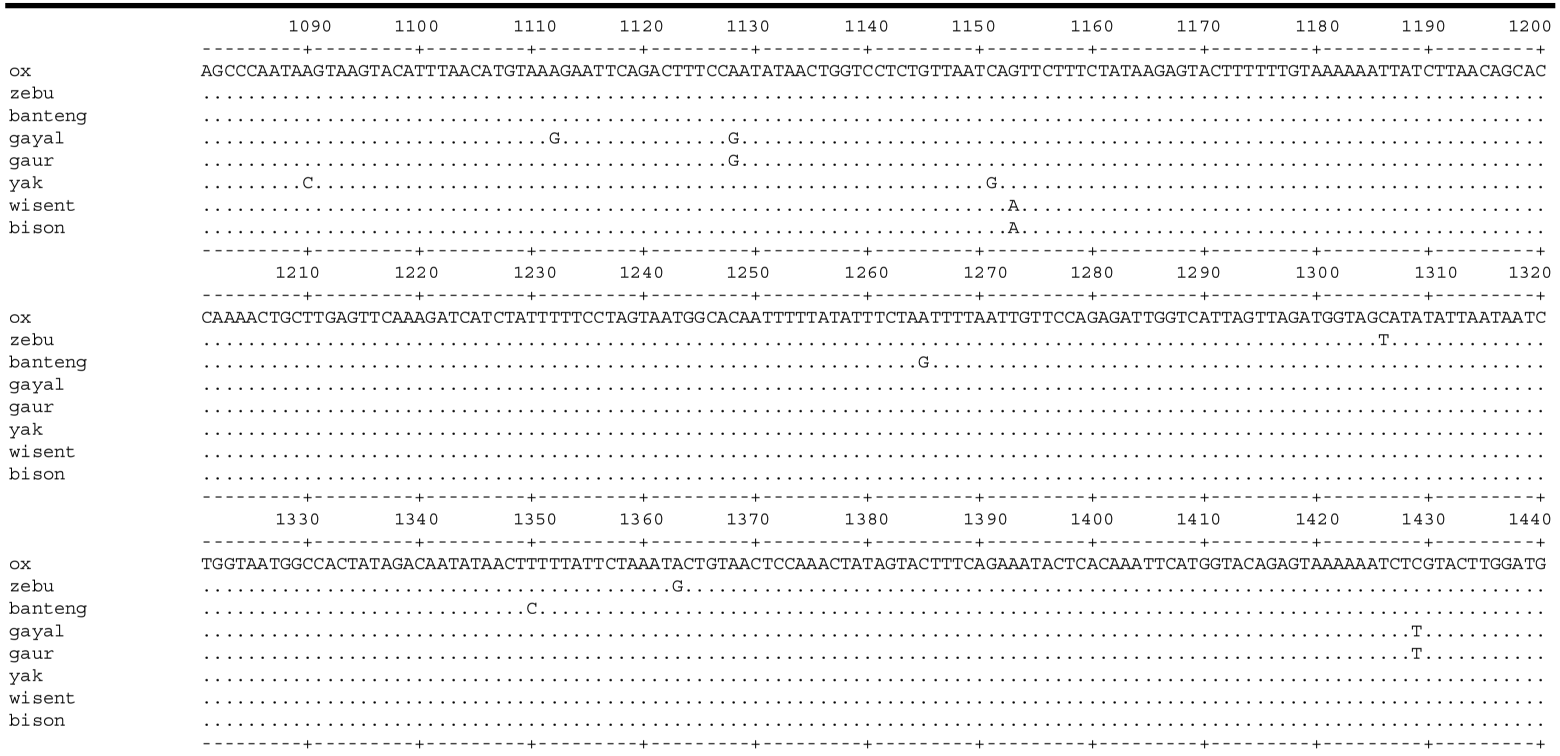
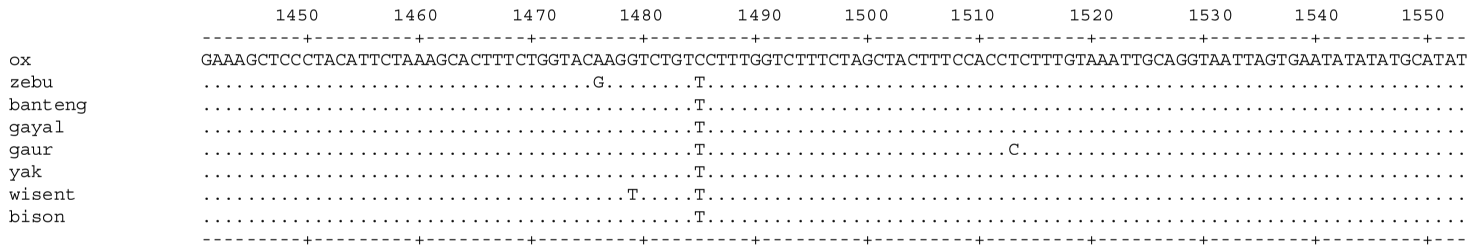





1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
ox AGCCCAATAAGTAAGTACATTTAACATGTAAAGAATTCAGACTTTCCAATATAACTGGTCCTCTGTTAATCAGTTCTTTCTATAAGAGTACTTTTTTGTAAAAAATTATCTTAACAGCAC
zebu
banteng
gayal
gaur
yak
wisent
bison
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320
ox CAAAACTGCTTGAGTTCAAAGATCATCTATTTTTCCTAGTAATGGCACAATTTTTATATTTCTAATTTTAATTGTTCCAGAGATTGGTCATTAGTTAGATGGTAGCATATATTAATAATC
zebu
banteng
gayal
gaur
yak
wisent
bison
1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440
ox TGGTAATGGCCACTATAGACAATATAACTTTTTATTCTAAATACTGTAACTCCAAACTATAGTACTTTCAGAAATACTCACAAATTCATGGTACAGAGTAAAAAATCTCGTACTTGGATG
zebu
banteng
gayal
gaur
yak
wisent
bison



ZFY

```
                10        20        30        40        50        60        70        80        90       100       110       120
                --------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
ox              CATGCTTTGCGAGATCTCAGAGGAAATTCCAGTATATATTCCGTAAAGGTGTTGTGATGGCATTTTAGTTGCTAGAATACTTGCACTGAATTTGAAAATACAGTTTTTAAGAATTAAGTG
zebu            ........................................................................................................................
banteng         ........................................................................................................................
gayal           ........................................................................................................................
gaur            ........................................................................................................................
yak             ........................................................................................................................
bison           ........................................................................................................................
wisent          ........................................................................................................................
                --------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                       130       140       150       160       170       180       190       200       210       220       230       240
                --------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
ox              ATATTCATGAATGATTTCCTTTCATAAAAAGAAACAGTTTCGAATCAGGATGTAATTCAGGAAAAACATTGAAAACTATCAGGTTACATTATTGGAAATGCAAGTAAATCACTAAAATGT
zebu            ........................................................................................................................
banteng         ..................................................A.....................................................................
gayal           ........................................................................................................................
gaur            ........................................................................................................................
yak             ...................................................................................................................G....
bison           ..........................................-.............................................................................
wisent          ........................................................................................................................
                --------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                       250       260       270       280       290       300       310       320       330       340       350       360
                --------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
ox              TAGGTTGGTTAACTTTAAATGAAAAAATTTAAAGTCTTTTTGTGTCAGCATGAAGCAGGCACTTTGACAGAGGAGCAGGACAAATACTACAGCTGTTAATTGAATATATTTCTTTTCTGT
zebu            ........................................................................................................................
banteng         ........................................................................................................................
gayal           ........................................................................................................................
gaur            ........................................................................................................................
yak             ........................................................................................................................
bison           ........................................................................................................................
wisent          ........................................................................................................................
                --------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                       370       380       390       400       410       420       430
                --------+---------+---------+---------+---------+---------+---------+-------
ox              ATAGCTTGTCTAGTGATTTGCACATTAGAATTTTTTATGGCTGTGATTGTAGTTTACTCAATTAATATAAATTTGAC
zebu            ...................................................................
banteng         ...................................................................
gayal           ...................................................................
gaur            ...................................................................
yak             ..........................G...........A.............................
bison           ............A..........................................................
wisent          ............A..........................................................
                --------+---------+---------+---------+---------+---------+---------+-------
```

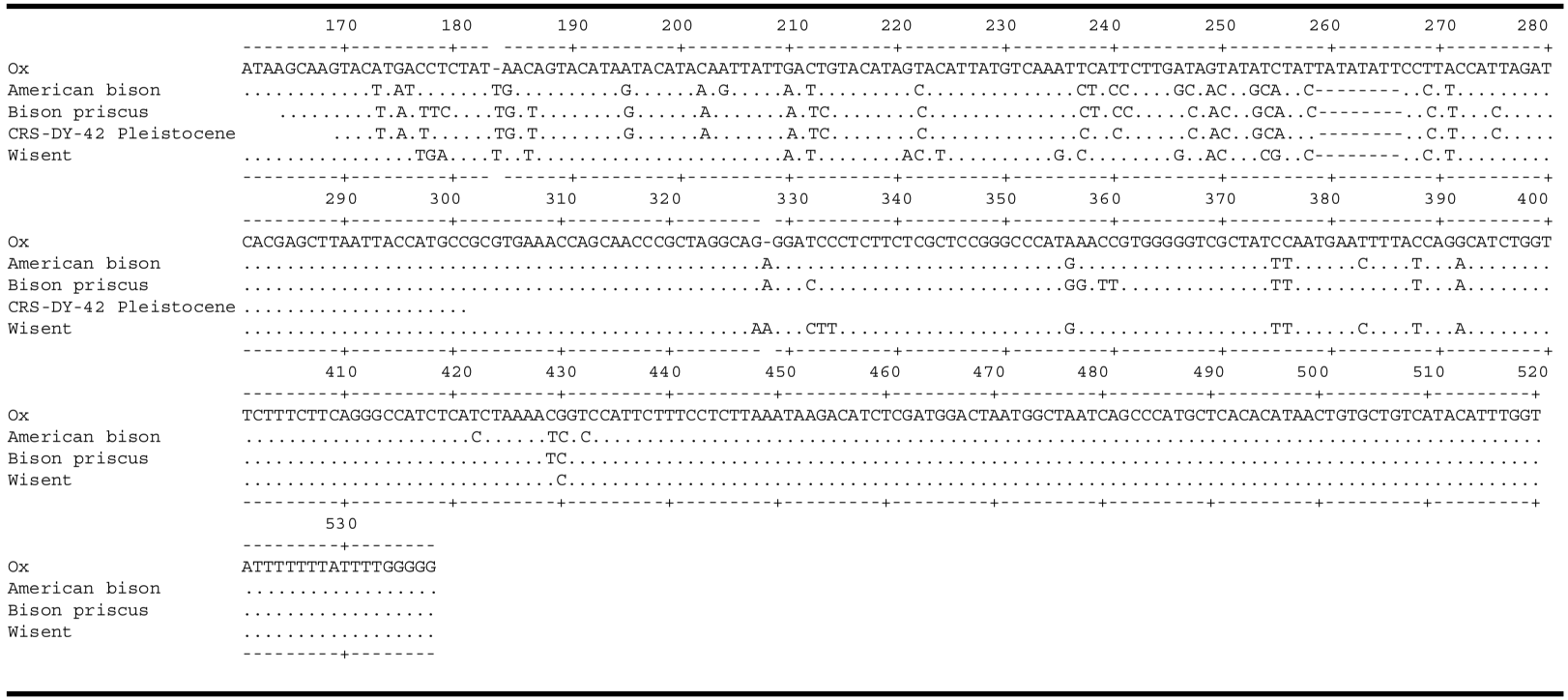


Figure 5. - Alignment of control regions from American bison (Bison bison), wisent (Bison bonasus), Bison priscus (CRS-SY-2, Nielsen-Marsh et al 2002, >55600 years old Genbank accession # AF538947) and a Pleistocene sample (CRS-DY-42, Willerslev et al 2003, Genbank accession # AF538947). The numbering is as in Figure 3.

CHAPTER 4

Organization and concerted evolution of the ampliconic Y-chromosomal TSPY gene of cattle

E.L.C.Verkaar, C.Zijlstra, E.M.van 't Veld, K.Boutaga, D.C.J.van Boxtel and J.A.Lenstra

Abstract

A typical feature of the male-specific part of mammalian Y-chromosomes (MSY) is the presence of several repeated genes and noncoding sequences. The multicopy *TSPY* (testis specific protein Y-encoded) gene encodes a nucleosome binding protein and has been found in several mammalian species. For cattle it has been estimated that 50 to 200 *TSPY* genes are dispersed over the MSY. We have studied the organization and evolution of the bovine *TSPY* genes. Analysis of BAC clones indicated that about half of the BAC clones contain a single *TSPY* gene. Other clones contain a cluster of at least four adjacent and truncated *TSPY* pseudogenes. Fluorescence *in situ* hybridization suggested that *TSPY* loci genes are mainly located on the short arm (Yp). Sequencing of *TSPY* segments from several BAC clones and from related bovine species indicated heterogeneity and concerted evolution of the intact *TSPY* genes.

1. Introduction

TSPY genes are located on the Y-chromosome of several mammalian species (Vogel *et al.* 1997b). *TSPY* encodes a testis-specific protein, which probably acts as nucleosome-binding transcription factor. Homology with the *SET/NAP/DENTT* oncogene family suggest a role in spermatogonic cell division and seminal oncogenesis (Lau 1999; Lau *et al.* 2000; Lau and Zhang 2000; Vogel and Schmidtke 1998).

Human *TSPY* is expressed in spermatogonia as early as during embryogenesis (Schnieders *et al.* 1996).With about 35 copies on Yp, it belongs to one of nine ampliconic gene families on the MSY (Dechend *et al.* 2000). Each copy is contained within the 20-kb unit of the *DYZ-5* tandem repeat (Dechend *et al.* 2000; Vogel *et al.* 1998). Other gene families on the human MSY are located on one of the eight large palindromic regions (Skaletsky *et al.* 2003).

A striking feature of *TSPY* is its variation of copy number in different mammals. Cattle is estimated to have 50 to 200 copies, while rat has only one gene copy (Dechend *et al.* 1998) and mouse a single pseudogene (Schubert *et al.* 2000). Like most other

ampliconic genes on the MSY, *TSPY* originates from insertions of autosomal sequences after the divergence of the sex-chromosomes (Charlesworth and Charlesworth 2000; Lahn and Page 1999; Marshall Graves 2000; Marshall Graves 2002).

In cattle heterogeneous *TSPY* mRNA transcripts have been identified, which align to 7 exons in genomic PCR products (Vogel *et al.* 1997b). The first part of bovine *TSPY* is related to Y-chromosomal repeats from cattle: *BRY.2* (Matthews and Reed 1992) and sheep (the OY family, Lord, Genbank entry U30305-U30307). In order to elucidate the organization and evolution of the bovine *TSPY* gene repeat family, we isolated and analysed BAC clones. We found two types of *TSPY* genes, *TSPY-M1* and *TSPY-M2*, but there was no evidence for a tandem-repeat organization. About half of the *TSPY* loci, denoted *TSPY-C*, contain a cluster of pseudogenes. Most *TSPY* loci are located on the short arm of the Y-chromosome. Comparison of *TSPY* genes from related bovine species indicate that *TSPY* is subject to concerted evolution.

2. Materials and Methods

2.1 Materials

Filters containing a 5 x coverage bovine BAC library (Zhu *et al.* 1999) were purchased from the Deutsches Resourcenzentrum für Genomforschung GmbH, Berlin, Germany (RZPD, **http://www.rzpd.de**). DNA from ox (*Bos taurus*), zebu (*Bos indicus*), gaur (*Bos gaurus*), yak (*Bos grunniens*), bison (*Bison bison*) and wisent (*Bison bonasus*) was isolated from whole blood using standard SDS/proteinase K extraction methods (Ciulla *et al.* 1988; Sambrook *et al.* 1989).

2.2 PCR amplification and sequencing

A *TSPY* probe was based on segment 2172-3189 of Genbank entry U75895 from the pRIAN bovine *TSPY* genomic sequence (Vogel *et al.* 1997b). This segment contains a part of exon 5, intron 5 and a part of exon 6 and is not similar to the *BRY.2* repeat (Matthews and Reed 1992). PCR was performed with 50 ng bovine genomic DNA, 50 ng of primers TSPY-5F (5'-TCA TCA GCG AAG ACG TGT GGG) and TSPY-6R (5'-AAG TGG CGA

GAG CGT GTT TTG G) in a total volume of 25 µl reaction buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP's and 1 U *Taq* polymerase (Promega, USA). The program consisted of 3 min predenaturation at 95°C, 30 cycles of 15 s at 95°C, 30 s at 58°C and 45 s at 72°C, followed by a final extension of 5 min at 72°C. Twenty ng of PCR product was labelled with [a^{32}P]dATP (Amersham, USA) using the MegaPrime labelling kit (Amersham).

Fragments of the *TSPY-M* gene in BAC clones were amplified with the primers derived from a pRIAN sequence (Vogel *et al.*, 1997; differences with the TSPY-M2 sequence in bold): exon 1a to exon 1b, 5'-AAG AGC TAG TCG GTC CCA CAG and 5'-**AAC TGA AC**G TAG G**T**C CTG **A**GG TT; within exon 1b, 5'-AC**C** GAG A**T**G TGG C**G**G G**G**A TC**C** or 5'-T**A**G ATG **C**CC TG**C** AAG CAC TGG and 5'-AA**A** CCA GGG ATG CCC TGG ATG; exon 1b to exon 2, 5'-T**A**G ATG **C**CC TG**C** AGG CAC TGG and 5'-TTC AAG TC**C** ATC ATG TAG C**C**G; exon 2 to exon 3, 5'-TCA AGT TTC C**A**T C**C**T GAT CAG and 5'- ATG TCA AGG TAA TAC TCC TTA; exon 3 to exon 4, 5'-TC**C** GC**C** GTC CC**G** CTG CAA GCT and 5'- CAA TCC TGT TCG ATT CTG GGC; exon 4 to exon 5, 5'-GT**C** GA**T** CCA CCC CAG TCC **A**CT and 5'-ACC CC**A** TCA C**C**A CAT GTG TTT. Other primers were derived from the TSPY-M1 sequence in BAC clone B016Q2: exon 1a to intron 2, 5'AAC CCC **C**GA CCT TCT TAG **C** and 5'-CAC TCC CCG CCA GGT CCC ATA C.

A 469-bp probe specific for the region downstream of the *UMN1605* microsatellite (Liu *et al.* 2002) was amplified with the primers 5'-AAT GAA GAC AGA GTG ATG AGC and 5'-CCC AAT CCT TAG CAG ATA GTT.

Sequencing of PCR products (15 µl, ca. 60 ng) was performed on an ABI Prism 310 sequencer (Applied Biosystems, USA). Genbank accession codes of *TSPY* fragments from different BAC clones and from ox, zebu, gaur, yak, bison are listed in the supplementary table 3.

2.3 BAC clones

Twenty thousand BAC clones on filter Q2 from RZPD library B750-199.2.215 were hybridized for 24 h at 60°C to the TSPY-5F/TSPY-6R probe in 30 ml hybridization buffer (2 mM Tris/EDTA, pH 8.3, 5% SDS, 0.1 % denatured herring sperm DNA). Membranes were washed twice for 15 min at 60°C in 2 X SSPE/0.1% SDS and once for 15 min at 60°C in 1 X SSPE/0.1% SDS. Subsequently, X-ray films were exposed overnight to the membranes. Clones hybridizing to the probe were grown overnight on LB-agar plates containing 0.1% chloramphenicol and then in 5 ml LB with 0.1% chloramphenicol for 16 h at 37°C. For Southern blotting, DNA was purified using the Qiagen high insert plasmid kit (Qiagen, USA). BAC DNA (20-50 ng) was digested in 10 µl with *Bam*HI (Amersham), *Pst*I (NEB, USA), *Pvu*II (NEB), *Kpn*I (NEB) and *Not*I (NEB) for 1 h at 37°C in the prescribed reaction buffer. Restriction fragments were separated in a 0.8% agarose/0.5 x TBE gel, 24 h at 25 V and blotted for 12 h in 0.4 M NaOH onto Hybond N+ nylon membrane (Amersham). The membrane was hybridized with the TSPY-5F/TSPY-6R probe as described above.

Subclones from BAC B016Q2 were generated after digestion with *Pst*I or *Bam*HI and sequenced with vector primers or by primer walking. In addition, PCR products were generated by exon-specific primers (see above) and sequenced. This resulted in a contig of 3332 bp (Genbank entry AY347578).

Subclones from BAC D0572Q2 obtained after digestion with *Pst*I or with *Hin*dIII and *Sst*I were selected with the TSPY-5F/TSPY-6R probe and with a $(CA)_{25}$ microsatellite probe. DNA from BAC D0572Q2 was sheared by sonification and subcloned in pcr4 blunt pTOPO vectors (Invitrogen, USA). DNA was extracted with a standard Qiaprep spin miniprep kit (Qiagen). Overnight cultures of 480 clones were spotted on a Hybond N membrane (Amersham), cross-linked by UV-irradiation (Stratalinker, Strategene) and hybridized consecutively to the TSPY-5F/TSPY-6R probe and overlapping probes contained in *Pst*I and a *Hin*dIII-*Sst*I subclones selected by the same TSPY-5F/TSPY-6R probe. Fifty clones were selected and sequenced (BaseClear, The Netherlands). Another *Pst*I subclone BAC D072Q2 selected by the $(CA)_{25}$ microsatellite probe contained the *UMN1605* microsatellite marker (Liu *et al.* 2002) and has been used to select other pTOPO clones.

Table 1. BAC clones from library RZPD B750-199.2.215, filter Q2, selected by hybridization to a *TSPY* probe. C, *TSPY-C* locus; M, *TSPY-M* locus; M1, *TSPY-M1* variant; M2; *TSPY-M2* variant. n.d., not determined

Clone	C /M	M1/M2	21 bp indel in exon 1b
A0157	C		
B0016	M	M2	deleted
B1726	C		
C0622	C	M2	deleted
D0572	C		
D2429	C		
E0134	S	M2	deleted
E1823	C		
F228	C		
F1944	M	n.d.	n.d.
F2334	M	M1	inserted
F0662	M	M1	inserted
G1018	C		
H1116	C		
I0562	C		
I0449	M	M1	deleted
I1730	M	M2	deleted
I1870	M	M1	inserted
J1732	M	n.d.	n.d.
N1838	M	M1	deleted
N1870	C		
O0151	C		
O0356	C		
O2444	M	M1	inserted
P0149	C		
P0086	M	n.d.	n.d.
P1116	C		
P1852	M	M2	deleted

2.4 Fluorescence in situ *hybridization*

In situ hybridization was performed with the TSPY-5F/TSPY-6R probe and the *UMN1605* PCR product (see above). Labelling with biotin-16-dUTP was performed by nick-translation. Labelled DNA was precipitated in the presence of a 50-fold excess of ssDNA (Sigma) and a 50-fold excess of fragmented total cattle DNA as competitor. FISH was performed on unbanded metaphase chromosomes as described by Zijlstra *et al.* (1997). The biotin labelled probe was visualized with fluorescein isothiocyanate (FITC)-conjugated avidin (Vector) and signals were amplified once using biotinylated goat-anti-avidin and avidin-FITC. Chromosomes were counterstained with propidiumiodide (PI). Double-target *in situ* hybridization of bovine metaphase spreads was performed by combining a digoxigenin labelled *TSPY* probe with a biotin labelled *UMN1605* probe or a biotin labelled *TSPY* probe with a digoxigenin labelled *UMN1605* probe, both at 10 ng/µl hybridization mix (Zijlstra *et al.* 1998). Hybridization and subsequent washing were performed as described (Bosma *et al.* 1996). The spreads were incubated with FITC conjugated avidin (DAKO, Copenhagen Denmark) and/or a monoclonal anti-digoxigenin antibody conjugated to tetramethyl rhodamine isothiocyanate (TRITC, DAKO, Copenhagen, Denmark). Spreads were counterstained with DAPI (Zijlstra *et al.* 1998). Images were examined using a Leica DMRA microscope equipped with the GENUS image analysis software of Applied Imaging. Images of FITC, TRITC, PI and/or DAPI fluorescence were recorded separately and subsequently merged by the GENUS software.

3. Results

3.1 Isolation and characterization of BAC clones

Screening of 20,000 BAC colonies with the TSPY-5F/6R probe specific for the region around the fifth intron of bovine *TSPY* yielded 64 positive colonies. Restriction digestion of 28 of these clones with various enzymes and subsequent Southern blot hybridization with the same probe revealed two types of patterns (Figure 1A). Thirteen clones exhibited a hybridization pattern with two *Pst*I fragments, *Bam*HI yielded only one fragment of 1.4 kb (not shown). The intensity of this fragment on an ethidium bromide-stained agarose gel

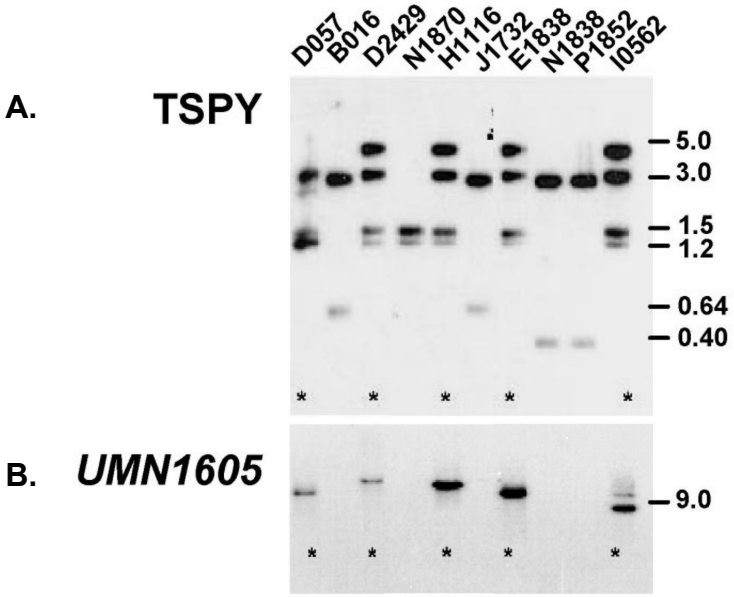


Figure 1. Southern blots of TSPY-positive BAC clones digested with PstI hybridized to (A) the TSPY-5F/TSPY-6R probe and (B) the UMN1605 probe. Asterisks indicate TSPY-C patterns.

Figure 2. Map of TSPY-M. The homology of TSPY-M and the upstream region contained in the genomic clone p0201 (Jakubiszka et al., 1994, dotted line) with the BRY.2 bovine repeat, the ovine OY.1, OY.4 and OY.9 repeats and with microsatellite loci are indicated.

indicated that these *TSPY* genes, designated as *TSPY-M* (monomer), are not repeated within the BAC clones. The hybridization patterns of the other BAC clones were more complex with at least three *Pst*I hybridizing fragments and are designated as *TSPY-C* (complex).

Sequencing revealed that the *TSPY-M* gene cloned in BAC B016Q2 is similar to the *TSPY* gene and cDNA described previously (Vogel *et al.* 1997b). The start codon in B016Q2 is 22 bp upstream of the ATG found in cDNA (Vogel *et al.* 1997b), but a deletion of 13 bp in exon 1a relative to the cDNA restores the reading frame. However, we found a 21-bp deletion and a stop codon in exon 1b, while the overall similarity is only 86%. Microsatellites in intron 2 and intron 5 are similar to the microsatellites *UMN0705* (Liu *et al*., 2002; 97 % similarity) and Bov 42.21 (Korokhov *et al.,* Genbank entry Y07737, 79 %).

We compared *TSPY-M* genes from 11 *TSPY-M* BAC clones by amplifying and sequencing the intron 5 – exon 6 regions. Sequences of four clones are highly similar to the sequence from BAC B016Q2 (97.2% or more), but differs at 15% of the positions from six other clones. These six sequences (Table 2) are almost identical to the pRIAN genomic PCR sequence and to the exon 6 region of cDNA (Vogel *et al.* 1997b). We denote the pRIAN-like sequences as *TSPY-M1* and the sequences resembling B016Q2 as *TSPY-M2*, respectively (Table 1). Amplification of exon 1b revealed that all *TSPY-M*2 and two *TSPY-M1* clones have the 21-bp deletion (Table 1).

Sequencing of subclones and partial assembly of the *TSPY-C* BAC clone D0572Q2 indicated at least four different truncated pseudogenes. A fragment spanning exon 2 to exon 5 is linked to a copy of the Y-chromosomal DNA *BTU39889* repeat (Grobet and Georges, Genbank entry U39889). At least two pseudogenes are coupled head-to-tail via an intron 5-exon 1b transition. The pseudogenes contain microsatellites at the same positions as in *TSPY-M*; two microsatellites in intron 2 were similar to INRA0182 (Vaiman *et al.* 1997, Genbank entry X73935, 90%) and Bov 42.3.7 (Korokhov *et al.,* Genbank entry Y07737, 85 %).

Selection of *Pst*I clones from BAC D0572Q2 with a microsatellite probe yielded a clone with a sequence similar to the Y-chromosomal microsatellite *UMN1605*. This sequence contains 56 bp similar to the initiation factor *EIF1AY* gene. Southern hybridization of BAC clones (Figure 1B) revealed that all *TSPY-C* clones tested contain the *UMN1605* sequence.

3.2 Fluorescence in situ *hybridization*

FISH staining of bovine metaphase spreads with the TSPY-5F/TSPY-6R probe, which does not cross-hybridize with the *BRY.2* repeat, generated several dots on the short arm (Yp) and a few dots on long arm (Yq) of the Y chromosome (Fig. 3). A probe specific for *UMN1605* predominantly stained a number of regions on Yq. Double staining revealed overlap of the *TSPY* and *UMN1605* signals only on Yp (Fig. 3). These results suggest that most *TSPY-M* and *TSPY-C* loci are on Yp, while most *UMN1605* copies are on Yq and are not associ ated with *TSPY*.

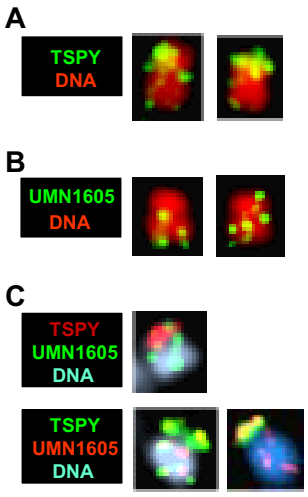


Figure 3. Y-chromosomes of male bovine lymphocytes were hybridized with (a) TSPY probe, (b) UMN1605 probe and (c) both probes simultaneously. Green signals correspond to FITC fluorescence, red signals to (a, b) propidium iodide or (c) TRITC fluorescence and blue signals to DAPI fluorescence.

3.3 Variation of TSPY-M1 in bovine species

Since the TSPY-5F and TSPY-6R primers have been designed on the basis of pRIAN (Vogel *et al.* 1997b), PCR products generated with genomic DNA had a *TSPY-M1*-like sequence. This sequence agrees well with the sequences from the *TSPY-M1* BAC clones (Table 2). At four positions heterogeneities could be identified by a double peak in the gel traces, both with a relatively low intensity and corresponding to differences between the clones and pRIAN. However, three other discrepancies between the genomic and the cloned sequences indicate the existence of *TSPY-M1* sequence variants not represented in the BAC clones.

Table 2. Variable position in a intron 5 - exon 6 segment amplicon of the *TSPY-M1* variant. The numbering corresponds to Genbank entry AY347578. Dots indicate identity with the ox (*Bos taurus*) genomic sequence

	000000111224556667777888
position	112389167015693991445279
	230782301730144183571410
pRIAN	C....AGGT.C-..A.C..GG....
BAC clones	
F0662Q2	.....AA.C..-...TA...T....
F2334Q2	...AA.C..-...TA...T....
I0449Q2	.....AA.C..-...TA...T....
I1870Q2	.....AA.C..-...TA...T....
N1838Q2	..AA.C..-...TA...T....
O2224Q2	..AA.T..C....A...T..T.
Genomic DNA	
Ox	CTCTTRRAYAT-AGACMGTCGACCG
Zebu	.....AG.T..-A...C........
Gaur	.....MG.T..-W...C........
Yak	.CTY.AA.TC.-..G.AA.Y..M.A
Bison	..T.CAA.T..-....AA...T..A
Wisent	..T.CAA.T..-....AA...T..A

A comparison of the sequences of bovine species (Table 2) revealed several mutations, most of which differentiate bison, wisent and yak from ox, zebu and gaur. Mutations shared by individual copies of a repeated sequence within a species indicate concerted evolution, by which copies within one species are more similar to each other than to copies of other species.

4. Discussion

4.1 The bovine TSPY loci

Isolation and sequencing of BAC clones has yielded new information about the structure and evolution of bovine *TSPY*. Sixty four positive clones out of 20.000 colonies with an average insert length of 105 kb (Zhu *et al.* 1999) correspond to 90 *TSPY* loci per Y-chromosome. Southern blot analysis of BAC clones revealed that these loci are divided equally over two categories, denoted as *TSPY-M* and *TSPY-C*. The *TSPY-M* clones contain a single gene, while the *TSPY-C* clones carry at least four truncated pseudogenes. By extrapolation the cattle Y-chromosome appears to contain 45 *TSPY-M* genes and 180 or more *TSPY* pseudogenes in the *TSPY-C* regions, which is in agreement with a previous estimate of 50 to 200 *TSPY* copies (Jakubiczka *et al.* 1993). Assuming a length of 4 kb for *TSPY-M* and 12 kb for *TSPY-C*, we estimate that the *TSPY* loci occupy at least 0.7 Mb or 1% of the bovine Y-chromosome. Since none of the BAC clones contained more than one *TSPY* locus, there is no evidence for a higher-order organization as observed for human *TSPY*.

Sequencing further differentiates two types of *TSPY-M*: *TSPY-M1*, which is most similar to the published pRIAN sequence and to cDNA (Vogel *et al.* 1997b), and *TSPY-M2*, which differs from *TSPY-M1* at 16 % of the nucleotide positions. However, exon 1b in clone B016Q2 as well as in clone P1852Q2 contains a stop codon. So the *TSPY-M2* copy in these clones either encode a truncated protein or a splice variant lacking intron 1b (Vogel *et al.* 1997b).

Both *TSPY-M* (Fig. 2) and *TSPY-C* contain microsatellite sequences in the third and last exons. The flanking sequences match the sequences of several microsatellite loci iden-

tified previously in four independent investigations (Liu *et al.* 2002). In addition, exon 6 from both the *TSPY-M* and the *TSPY-C* loci matched to subclones of the cosmid carrying IDVGA50 (Mezzelani *et al.*, Genbank entries X89420 and X89425), while *TSPY-C* is associated with *UMN0605*. This suggests that a significant portion of the microsatellites on the bovine Y-chromosome are contributed by *TSPY* or *TSPY*-related loci. Other significant matches were found to segments contained within other Y-chromosomal microsatellite loci (INRA008, INRA062, INRA109, UMN2405 and UMN2611), which indicates exchange of sequence segments between Y-chromosomal loci.

FISH experiments suggest that most *TSPY* loci are predominantly on Yp, which also contains *ZFY* (Xiao *et al.* 1998) and *AMELY* (Liu *et al.* 2002). The granular FISH patterns indicate a clustering of *TSPY* loci and were also observed on both Y-chromosomal arms after staining with a *BRY.2* probe (results not shown). The previously described dispersal of *TSPY* genes over the whole Y-chromosome (Vogel *et al.* 1997a) is probably explained by a cross-hybridization of *TSPY* with the related *BRY.2* Y-chromosomal repeat (Matthews and Reed 1992).

4.2 Y-chromosomal evolution

As indicated in Figure 2, different parts of *TSPY* as well as the region upstream of *TSPY* are related to the bovine Y-chromosomal repetitive element *BRY.2* (Matthews and Reed 1992) and to the ovine elements OY.1, OY.4 and OY.11 (Lord, Genbank entries U30305, U30307 and U30378, respectively). *BRY.2* is estimated to be present in 1200 copies. Thus by truncations, translocations and amplifications, various *TSPY*-related repetitive elements have emerged on the bovine as well as the ovine Y-chromosome by truncation, degeneration and amplification of TSPY sequences (Figure 4). The finding that *TSPY* matches with segments from several microsatellite loci (see above) also reinforces the notion that rearrangements and duplications within the MSY are frequent.

The present observations illustrate the rapid and partially degenerative evolution of the mammalian Y-chromosome (Marshall Graves 2000; Marshall Graves 2002), which is shaped by a lack of recombination (Charlesworth and Charlesworth 2000), by an accumulation of genes or gene variants that are advantageous for males (Lahn and Page 1999; Lahn *et al.* 2001; Marshall Graves 2000; Marshall Graves 2002; Rice and Holland 1997; Wang

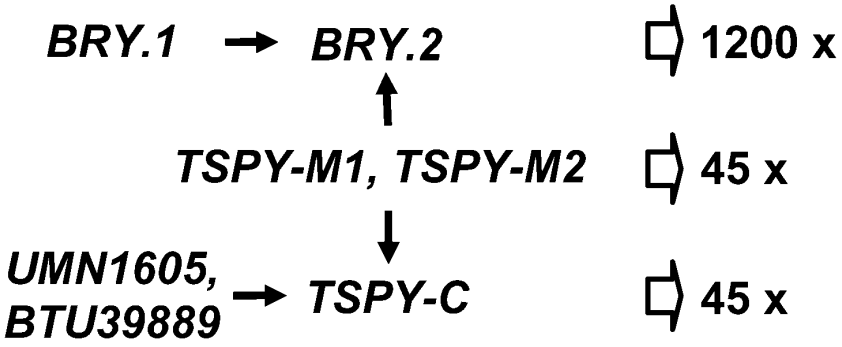


Figure 4. *TSPY*-related Y-chromosomal repetitive elements in cattle. *BRY.1* (Matthews and Reed, 1992) and *TSPY-M* both contribute to the *BRY-2* repeat, while the *TSPY-C* loci also contain a copy of the *UMN1605* microsatellite (Liu *et al.*, 2002) and of the *BTU39889* repeat (Grobet, unpublished).

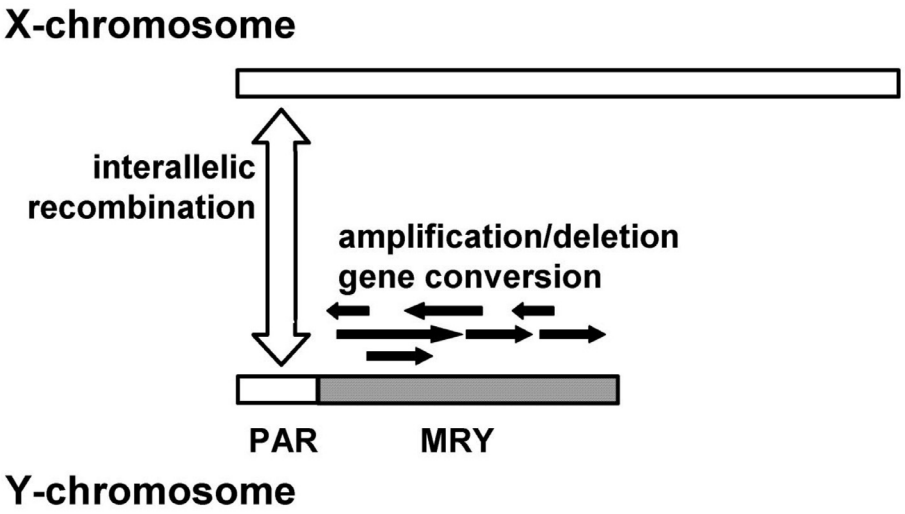


Figure 5. Horizontal exchange of genetic information within the Y-chromosome and vertical meiotic recombination between the X and Y-chromosomes.

et al. 2001) and by genetic conflicts with X-chromosomal or autosomal genes (Amos and Harwood 1998; Hurst 1998; Rice and Holland 1997).

Differences between the repeated *TSPY-M* sequences from related bovine species suggest concerted evolution, which implies a mechanism for homogenization of members of a gene family. Rozen *et al.* (2003) described gene conversion within large palindromic regions of the human Y-chromosome. Another mechanism by which copies within one species are more similar to each other than to copies of another species is amplification and deletion of sequence variants according to the birth-and-death model (Rooney *et al.* 2002). It is not clear yet which mechanism operates on the tandemly repeated human *TSPY* or on the bovine *TSPY* loci, which are more dispersed. However, the rapid horizontal changes in the MSY have taken place within the 1 Myr divergence time that has separated bison and taurine cattle. This is comparable to the dynamic evolution of centromeric satellite DNA (Nijman and Lenstra 2001). Similar findings have been observed for simian *TSPY* (Tosi *et al.* 2000).

A likely consequence of the high density of *TSPY* and other Y-chromosomal repeats on the MSY is a high frequency of intrachromosomal interactions. This may facilitate horizontal evolution by amplifications, deletions and gene conversion (Fig. 5) and contrasts to the vertical exchange of genetic information by meiotic recombination of the PAR and the X-chromosome.

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References

Amos, W., and J. Harwood, 1998 Factors affecting levels of genetic diversity in natural populations. Philos Trans R Soc Lond B Biol Sci **353:** 177-186.

Bosma, A. A., N. A. de Haan, C. H. Mellink, M. Yerle and C. Zijlstra, 1996 Chromosome homology between the domestic pig and the babirusa (family Suidae) elucidated with the use of porcine painting probes. Cytogenet Cell Genet **75:** 32-35.

Charlesworth, B., and D. Charlesworth, 2000 The degeneration of Y chromosomes. Philos Trans R Soc Lond B Biol Sci **355:** 1563-1572.

Ciulla, T. A., R. M. Sklar and S. L. Hauser, 1988 A simple method for DNA purification from peripheral blood. Anal Biochem **174:** 485-488.

Dechend, F., S. Schubert, I. Nanda, T. Vogel, M. Schmid *et al.*, 1998 Organization and expression of rat Tspy. Cytogenet Cell Genet **83:** 270-274.

Dechend, F., G. Williams, B. Skawran, S. Schubert, M. Krawczak *et al.*, 2000 TSPY variants in six loci on the human Y chromosome. Cytogenet Cell Genet **91:** 67-71.

Hurst, L. D., 1998 Selfish genes and meiotic drive. Nature **391:** 223.

Jakubiczka, S., F. Schnieders and J. Schmidtke, 1993 A bovine homologue of the human TSPY gene. Genomics **17:** 732-735.

Lahn, B. T., and D. C. Page, 1999 Four evolutionary strata on the human X chromosome. Science **286:** 964-967.

Lahn, B. T., N. M. Pearson and K. Jegalian, 2001 The human Y chromosome, in the light of evolution. Nat Rev Genet **2:** 207-216.

Lau, Y. F., 1999 Gonadoblastoma, testicular and prostate cancers, and the TSPY gene. Am J Hum Genet **64:** 921-927.

Lau, Y. F., P. M. Chou, J. C. Iezzoni, J. A. Alonzo and L. G. Komuves, 2000 Expression of a candidate gene for the gonadoblastoma locus in gonadoblastoma and testicular seminoma. Cytogenet Cell Genet **91:** 160-164.

Lau, Y. F., and J. Zhang, 2000 Expression analysis of thirty one Y chromosome genes in human prostate cancer. Mol Carcinog **27:** 308-321.

Liu, W. S., P. Mariani, C. W. Beattie, L. J. Alexander and F. A. Ponce De Leon, 2002 A radiation hybrid map for the bovine Y Chromosome. Mamm Genome **13:** 320-326.

Marshall Graves, J. A., 2000 Human Y chromosome, sex determination, and spermatogenesis- a feminist view. Biol Reprod **63:** 667-676.

Marshall Graves, J. A., 2002 The rise and fall of SRY. Trends Genet **18:** 259-264.

Matthews, M. E., and K. C. Reed, 1992 Sequences from a family of bovine Y-chromosomal repeats. Genomics **13:** 1267-1273.

Nijman, I. J., and J. A. Lenstra, 2001 Mutation and recombination in cattle satellite DNA: a feedback model for the evolution of satellite DNA repeats. J Mol Evol **52:** 361-371.

Rice, W. R., and B. Holland, 1997 The enemies within: intergenomic conflict, interlocus contest evolution (ICE), and the intraspecific Red Queen. Behav Ecol Sociobiol**:** 1-10.

Rooney, A. P., H. Piontkivska and M. Nei, 2002 Molecular evolution of the nontandemly repeated genes of the histone 3 multigene family. Mol Biol Evol **19:** 68-75.

Rozen, S., H. Skaletsky, J. D. Marszalek, P. J. Minx, H. S. Cordum *et al.*, 2003 Abundant gene conversion between arms of palindromes in human and ape Y chromosomes. Nature **423:** 873-876.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory press, Cold Spring Harbor, USA.

Schnieders, F., T. Dork, J. Arnemann, T. Vogel, M. Werner *et al.*, 1996 Testis-specific protein, Y-encoded (TSPY) expression in testicular tissues. Hum Mol Genet **5:** 1801-1807.

Schubert, S., F. Dechend, B. Skawran, M. Krawczak and J. Schmidtke, 2000 Molecular evolution of the murine tspy genes. Cytogenet Cell Genet **91:** 239-242.

Skaletsky, H., T. Kuroda-Kawaguchi, P. J. Minx, H. S. Cordum, L. Hillier *et al.*, 2003 The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature **423:** 825-837.

Tosi, A. J., J. C. Morales and D. J. Melnick, 2000 Comparison of Y Chromosome and mtDNA Phylogenies Leads to Unique Inferences of Macaque Evolutionary History. Mol Phylogenet Evol **17:** 133-144.

Vaiman, D., L. Schibler, A. Oustry, A. Schmitz, J. P. Furet *et al.*, 1997 A cytogenetically anchored genetic map of bovine chromosome 1 obtained by integrating flow-sorted chromosome-derived microsatellite markers into the international bovine map. Cytogenet Cell Genet **79:** 204-207.

Vogel, T., H. Boettger-Tong, I. Nanda, F. Dechend, A. I. Agulnik *et al.*, 1998 A murine TSPY. Chromos Res **1:** 35-40.

Vogel, T., S. Borgmann, F. Dechend, W. Hecht and J. Schmidtke, 1997a Conserved Y-chromosomal location of TSPY in Bovidae. Chromos Res **5:** 182-185.

Vogel, T., F. Dechend, E. Manz, C. Jung, S. Jakubiczka *et al.*, 1997b Organization and expression of bovine TSPY. Mammal Genome **8:** 491-496.

Vogel, T., and J. Schmidtke, 1998 Structure and function of TSPY, the Y-chromosome gene coding for the "testis-specific protein". Cytogenet Cell Genet **80:** 209-213.

Wang, P. J., J. R. McCarrey, F. Yang and D. C. Page, 2001 An abundance of X-linked genes expressed in spermatogonia. Nat Genet **27:** 422-426.

Xiao, C., K. Tsuchiya and S. Sutou, 1998 Cloning and mapping of bovine ZFX gene to the long arm of the X-chromosome (Xq34) and homologous mapping of ZFY gene to the distal region of the short arm of the bovine (Yp13), ovine (Yp12-p13), and caprine (Yp12-p13) Y chromosome. Mamm Genome **9:** 125-130.

Zhu, B., J. A. Smith, S. M. Tracey, B. A. Konfortov, K. Welzel *et al.*, 1999 A 5x genome coverage bovine BAC library: production, characterization, and distribution. Mamm Genome **10:** 706-709.

Zijlstra, C., R. Davoli, L. Fontanesi, P. Zambonelli, A. A. Bosma *et al.*, 1998 Isolation and localization of the skeletal myosin heavy chain 2X gene on pig chromosome 12q1.4-q1.5. Mamm Genome **9:** 412-413.

Supplement Chapter 4

Table 3. Genbank accession codes of *TSPY* fragments from different BAC clones and bovine species

BAC clones	
Exon 1a to exon 1b-B016	AY347579
Exon 1a to exon 1b-F2334	AY347580
Exon 1b F0662	AY347593
Exon 1b O2444	AY347594
Exon 1b F2334	AY347595
Exon 1b B016	AY347596
Exon 1b P1852	AY347597
Exon 1b I0449	AY347598
Exon 1b C0662	AY347599
Exon 5 to exon 6-O2444	AY347581
Exon 5 to exon 6-F2334	AY347582
Exon 5 to exon 6-I1870	AY347583
Exon 5 to exon 6-I0449	AY347584
Exon 5 to exon 6-N1838	AY347585
Exon 5 to exon 6-F0662	AY347586
Exon 5 to exon 6-C0622	AY347600
Exon 5 to exon 6-E0134	AY347601
Exon 5 to exon 6-P1852	AY347602
Exon 5 to exon 6-I1730	AY347603
Species	
Exon5 to exon 6 Ox	AY347587
Exon 5 to exon 6 Zebu	AY347588
Exon 5 to exon 6 Gaur	AY347589
Exon 5 to exon 6 Yak	AY347590
Exon 5 to exon 6 Bison	AY347591
Exon 5 to exon 6 Wisent	AY347592

CHAPTER 5

Summarizing discussion

The research described in this thesis is focused on the structure and evolution of the bovine Y-chromosome and the use of paternal markers in molecular diagnostics. The Y-chromosome has emerged together with the X chromosome early during the evolution of the mammals by differentiation of a pair of autosomes (Marshall Graves and Shetty 2001). The Y-chromosome is now the male chromosome and is transmitted from father to son. It starts the development of male properties in mammals primarily by the action of the gene *SRY* (Cotinot *et al.* 2002; Mackay 2000). Several other genes on the Y-chromosome are involved in spermatogenesis (Lahn *et al.* 2001; Quintana-Murci *et al.* 2001).

The **first chapter** is a general introduction and starts out with a description of the well-characterized human Y-chromosome, which may serve as model for its mammalian homologues. Sequencing of the complete Y-chromosome revealed 27 distinct protein-coding genes (Skaletsky *et al.* 2003). Two genes have been transposed after the human-chimpanzee divergence from the X-chromosome. Eighteen genes are denoted as 'X-degenerate': single-copy genes with a X-chromosomal homologue and, with a few exceptions, expressed ubiquitously. Nine 'ampliconic' genes are present in more than one copy, do not have an X-chromosomal homologue and are expressed in the testis (Lahn and Page 1997; Quintana-Murci *et al.* 2001). Only the mode of action of the X-degenerate *SRY*, which acts as transcription factor, has been studied into some detail (Cotinot *et al.* 2002).

It is illustrative for modern molecular biology that much of our knowledge about the mammalian Y-chromosome has been derived from the studies on three different mammals:

- Analysis of the *wallaby* sex chromosomes have revealed which genes have been conserved since the differentiation of the proto-X-Y chromosomes, while the localization of a group of genes on the autosomal region 5q of Wallaby indicated the source of other genes on the placental sex chromosomes (Graves *et al.* 1998).
- Y-chromosomal abnormalities and microdeletions in *men* demonstrate the role of Y-chromosomal genes in male development and fertility (Quintana-Murci *et al.* 2001).
- Transgenic *mice* contribute by linking a Y-chromosomal gene directly to a dominant phenotype. In addition, *in situ* hybridization with coupes of mouse embryos (Sinclair *et al.* 2002) allow detailed studies of gene expression as dependent on developmental stage.

In the near future, EST sequencing or SAGE strategies may identify other genes relevant for the male properties. In addition, expression studies with microarrays have the potential

to unravel the regulatory pathways initiated by *SRY* and other Y-chromosomal transcription factors. These studies may very well have practical applications if this leads to the development of drugs interfering in the spermatogenesis for use as male contraceptive.

A survey of the available data on gene content and repetitive elements of the cattle Y-chromosome indicates that the human and bovine Y-chromosomes have several, but not all genes in common. One of the most interesting ampliconic bovine Y-chromosomal genes is the repetitive gene *TSPY*, which has a variable copy number in different mammals (Jakubiczka *et al.* 1993; Vogel *et al.* 1997a; Vogel *et al.* 1997b). Hopefully, sequencing of the complete cattle genome in the near future will generate much more data on the bovine Y-chromosome, although these studies tend to neglect the repetitive genes (Lander *et al.* 2001).

We place the information on the structure and function of the Y-chromosome in the context of its unique evolution. First, it is more than any other chromosome susceptible to degeneration because it does not recombine with a homologous chromosome during meiosis (Charlesworth and Charlesworth 2000). Second, the gene content of Y-chromosomes often changes by insertions, amplifications or deletions (Lahn *et al.* 2001). Third, male-benefit alleles are selected rapidly by dominant transmission (Rice and Holland 1997). Finally, the Y-chromosome becomes easily involved in genetic conflicts and may be very well involved in the maintenance of the sex ratio (Hurst 1998; Hurst *et al.* 1996).

Genetic polymorphisms on the Y-chromosome have become useful as markers of the paternal lineage for tracing descent and inheritance. Recent studies of human evolution have shown the power of Y-chromosomal markers, which also offer perspectives in forensic analysis (de Knijff 2000; Quintana-Murci *et al.* 2001; Verkaar *et al.* 2003a; Verkaar *et al.* 2003b). For cattle only few polymorphic Y-chromosomal markers are available (Edwards *et al.* 2000; Liu *et al.* 2002). However, we anticipate that in the coming years bovine Y-chromosomal SNP's will be identified, which will allow a further elucidation of the history of domestication. We also survey the use of other genetic and phylogenetic DNA markers in men and cattle: various types of variation in nuclear DNA and mitochondrial DNA as marker of the maternal lineage (Bradley *et al.* 1996; Janecek *et al.* 1996).

The potential of these markers is illustrated by the test methods described in **Chapter 2**, which consists of three parts. In **Chapter 2a,** the development of two comple-

mentary methods is described: a mitochondrial PCR-RFLP and satellite fragment length polymorphism or SFLP (Nijman *et al.* 1999) for the detection and differentiation of the species origin of beef. SFLP is especially relevant for the identification of samples from animals of hybrid origin (Verkaar *et al.* 2000). **Chapter 2b** describes assays based on mutations in the paternally inherited Y-chromosomes of different bovine species for the detection of male introgression. The practical value of these assays is demonstrated by the analysis of bulls from a hybrid bison-wisent population in Belgium and by the detection of the banteng origin of bulls from the Indonesian Madura zebu breed (Verkaar *et al.* 2003b). This information is most relevant for ongoing conservation efforts (H.Udo, personal communication). In **Chapter 2c** we describe a real-time PCR method for a sensitive detection of the interspersed repeat Bov-A2 (Lenstra *et al.* 1993). The element occurs frequently in the genomes of all ruminants and is a suitable target for the DNA detection of ruminant material in feedstuff (Tajima *et al.* 2002). The use of meat-and-bone meal from sheep in feedstuff has probably caused the emergence of BSE in cattle. Subsequently, consumption of beef from BSE-infected cattle has in men led to fatal cases of a juvenile form of Creutzfeldt-Jakob's disease (Will 1999). Therefore the use of material of ruminants or other mammals in feedstuff for cattle is now forbidden. We show that our assay has the required specificity and sensitivity for detection of cattle, sheep or goat DNA in feedstuff that has been subject to an extreme heat treatment. With a detection level of about 10 fg DNA our method has a higher sensitivity than any method described sofar. We have provided proof-of-concept and after a further validation with field samples our method may contribute to the safety of feedstuff.

In the new Framework 6 research program of the European community food traceability has a high priority. In addition to methods as described in Chapter 2, genetic markers are likely to be used for tracing the breed or region of origin. Although mitochondrial DNA, microsatellites as well as AFLP markers are variable also within breeds, assignment of samples to breeds on the basis of a large panel of markers has been shown to be feasible (Rosenberg *et al.* 2001). Y-chromosomal markers can only be tested in products of male origin, but would for ruminants offer the advantage of a relatively low within-breed variability because of the small male effective-population size.

In **Chapter 3** we have used the mitochondrial and Y-chromosomal variation for elucidating the phylogeny of the *Bovini* or cattle-like species (Verkaar *et al.* 2003a). As expected, we found a clear relationship of ox and zebu, but also of gaur, gayal and banteng and of yak and American bison. However, we found that the mitochondrial DNAs from American bison and wisent (European bison) were quite different, although their hybrid offspring is fully fertile and their Y-chromosomal sequences are clearly related. Thus it appears that wisent has via the paternal lineage another origin than via the maternal lineage. Theoretically this may be explained by lineage sorting: a coexistence of two mitochondrial types in the wisent-bison lineage and the fixation of different types in the separate lineages leading to the individual species. More plausible is that the wisent emerged by male-mediated introgression of American bison or the extinct Pleistocene steppe wisent (*Bison priscus*) in an ancestral aurochs population during the late Pleistocene-early Holocene (10.000 to 20.000 years ago). After a few generations of introgression the phenotype as well as the Y-chromosome of the population became bison-like, but the original mitochondrial DNA has been maintained. This would explain the sudden and mysterious appearance of the wisent in the fossil record (Pucek 1986).

In general, speciation can be *allopatric* (divergence after separation of populations by geographical barriers, like the African and Indian elephants), *sympatric* (differentiation within the same region, like the Galápagos finches studied by Darwin) or *parapatric* (differentiation during separation by a hybrid zone). For the speciation mechanism via male introgression that has led to the emergence of the wisent and which has observed for American deer (Cathey *et al.* 1998), ibex (Pidancier et al., unpublished) and macaque species (Tosi *et al.* 2000), we propose the term *transpatric*. The transpatric speciation of wisents is still hypothetical, but can be put to test by DNA analysis of fossil remains of bisons and aurochs (Nielsen-Marsh *et al.* 2002). Alternatively, a detailed comparison of the genomes of wisent, bison and cattle may reveal traces of an auroch ancestry of the wisent.

More detailed information of the structure of the cattle Y-chromosome will be required for a further elucidation of its function as modulated by a dynamic evolution (see above). For this a characterization of Y-chromosomal repetitive elements, as has also been carried out for the human Y-chromosome (Tilford *et al.* 2001), will be essential. We have contributed to this in **Chapter 4** by an analysis of the multicopy *TSPY* gene (Verkaar *et al.*

2003c). Because of its estimated copy number of 50 to 200 and testis-specific expression it appears to have a prominent role in male development (Vogel *et al.* 1998; Vogel and Schmidtke 1998). By selection and analysis of BAC clones we arrived at an estimate of 180 copies or more. Fluorescent *in situ* hybridization (FISH) indicated that most of the *TSPY* loci are on the short arm (Yp) of the bovine Y-chromosome. However, the majority of the *TSPY* sequences are contained in *TSPY-C* loci, a cluster of truncated pseudogenes. Sequence analysis indicated two types of full-length *TSPY* genes, *TSPY-M1* and *TPSY-M2*, but it is not clear yet how many of these are expressed. In contrast to the tandem repeat organization of human *TSPY*, the bovine *TSPY-M* sequences appear to be dispersed, since we found only one *TSPY* locus per BAC clone of 105 kb. It may be hypothesized that multicopy genes play a special role during the evolution of the Y-chromosome by facilitating a flexible response in genetic conflicts to mutations in antagonistic loci. This may be explored further by comparing functional properties of active *TSPY* variants in cattle and related species.

Comparison of active *TSPY* copies of different bovine species yielded evidence for concerted evolution, since *TSPY* copies of taurine cattle were more similar to each other than to copies of other species. This can be explained either by gene conversion or by a birth-and-death process (Rooney *et al.* 2002), in which the amplification of a few copies and the deletion of others maintains the homogeneity. Concerted evolution in Y-chromosomal repeats has so far not been studied in detail (Tosi *et al.* 2000). We predict that other Y-chromosomal repetitive elements, genes as well as high-copy non-coding sequences, are also subject to rapid homogenization. In this respect, the Y-chromosomal repeats resemble the autosomal centromeric satellite DNA (Nijman and Lenstra 2001). A promising technique for the analysis of the rapid evolution of the Y-chromosome is fluorescent *in situ* hybridization of DNA fibers, which allows a high resolution localization of repetitive sequences (Conrad *et al.* 1996).

We conclude that a further study of the mammalian Y-chromosome will prove rewarding because of its relevance for structural and functional genomics, for evolutionary and reproductive biology, and for phylogeographic and forensic analysis.

References

Bradley, D. G., D. E. MacHugh, P. Cunningham and R. T. Loftus, 1996 Mitochondrial diversity and the origins of African and European cattle. Proc Natl Acad Sci U S A **93:** 5131-5135.

Cathey, A. C., J. W. Bickham and J. C. Patto, 1998 Introgressive hybridization and nonconcordant evolutionary history of maternal and paternal lineages in north-american deer. Evolution **52:** 1224-1229.

Charlesworth, B., and D. Charlesworth, 2000 The degeneration of Y chromosomes. Philos Trans R Soc Lond B Biol Sci **355:** 1563-1572.

Conrad, C., T. Hierl, B. Glaser, K. Taylor, S. Zeitler *et al.*, 1996 High-resolution fluorescence in situ hybridization of RBM- and TSPY-related cosmids on released Y chromatin in humans and pygmy chimpanzees. Chromosome Res **4:** 201-206.

Cotinot, C., E. Pailhoux, F. Jaubert and M. Fellous, 2002 Molecular genetics of sex determination. Semin Reprod Med **20:** 157-168.

de Knijff, P., 2000 Messages through bottlenecks: on the combined use of slow and fast evolving polymorphic markers on the human Y chromosome. Am J Hum Genet **67:** 1055-1061.

Edwards, C. J., C. Gaillard, D. G. Bradley and D. E. MacHugh, 2000 Y-specific microsatellite polymorphisms in a range of bovid species. Anim Genet **31:** 127-130.

Graves, J. A., C. M. Disteche and R. Toder, 1998 Gene dosage in the evolution and function of mammalian sex chromosomes. Cytogenet Cell Genet **80:** 94-103.

Hurst, L. D., 1998 Selfish genes and meiotic drive. Nature **391:** 223.

Hurst, L. D., A. Atlan and B. O. Bengtsson, 1996 Genetic conflicts. Q Rev Biol **71:** 317-364.

Jakubiczka, S., F. Schnieders and J. Schmidtke, 1993 A bovine homologue of the human TSPY gene. Genomics **17:** 732-735.

Janecek, L. L., R. L. Honeycutt, R. M. Adkins and S. K. Davis, 1996 Mitochondrial gene sequences and the molecular systematics of the artiodactyl subfamily bovinae. Mol Phylogenet Evol **6:** 107-119.

Lahn, B. T., and D. C. Page, 1997 Functional coherence of the human Y chromosome. Science **278:** 675-680.

Lahn, B. T., N. M. Pearson and K. Jegalian, 2001 The human Y chromosome, in the light of evolution. Nat Rev Genet **2:** 207-216.

Lander, E. S., L. M. Linton, B. Birren, C. Nusbaum, M. C. Zody *et al.*, 2001 Initial sequencing and analysis of the human genome. Nature **409:** 860-921.

Lenstra, J. A., J. A. van Boxtel, K. A. Zwaagstra and M. Schwerin, 1993 Short interspersed nuclear element (SINE) sequences of the Bovidae. Anim Genet **24:** 33-39.

Liu, W. S., P. Mariani, C. W. Beattie, L. J. Alexander and F. A. Ponce De Leon, 2002 A radiation hybrid map for the bovine Y Chromosome. Mamm Genome **13:** 320-326.

Mackay, S., 2000 Gonadal development in mammals at the cellular and molecular levels. Int Rev Cytol **200:** 47-99.

Marshall Graves, J. A., and S. Shetty, 2001 Sex from W to Z: evolution of vertebrate sex chromosomes and sex determining genes. J Exp Zool **290:** 449-462.

Nielsen-Marsh, C. M., P. H. Ostrom, H. Gadhi, B. Shapiro, A. Cooper *et al.*, 2002 Sequence preservation of osteocalcin and mitochondrial DNA in bison bones older than 55 ka. Geology **30:** 1099-1102.

Nijman, I. J., D. G. Bradley, O. Hanotte, M. Otsen and J. A. Lenstra, 1999 Satellite DNA polymorphisms and AFLP correlate with Bos indicus-taurus hybridization. Anim Genet **30:** 265-273.

Nijman, I. J., and J. A. Lenstra, 2001 Mutation and recombination in cattle satellite DNA: a feedback model for the evolution of satellite DNA repeats. J Mol Evol **52:** 361-371.

Pucek, Z., 1986 Bison bonasus (Linnaeus, 1758)-Wisent. Pp. 278-315 in Handbuch der Säugetier Europas, Aula Verlag, GmbH, Wiesbaden.

Quintana-Murci, L., C. Krausz and K. McElreavey, 2001 The human Y chromosome: function, evolution and disease. Forensic Sci Int **118:** 169-181.

Rice, W. R., and B. Holland, 1997 The enemies within: intergenomic conflict, interlocus contest evolution (ICE), and the intraspecific Red Queen. Behav Ecol Sociobiol**:** 1-10.

Rooney, A. P., H. Piontkivska and M. Nei, 2002 Molecular evolution of the nontandemly repeated genes of the histone 3 multigene family. Mol Biol Evol **19:** 68-75.

Rosenberg, N. A., T. Burke, K. Elo, M. W. Feldman, P. J. Freidlin *et al.*, 2001 Empirical evaluation of genetic clustering methods using multilocus genotypes from 20 chicken breeds. Genetics **159:** 699-713.

Sinclair, A., C. Smith, P. Western and P. McClive, 2002 A comparative analysis of vertebrate sex determination. Novartis Found Symp **244:** 102-111; discussion 111-104, 203-106, 253-107.

Skaletsky, H., T. Kuroda-Kawaguchi, P. J. Minx, H. S. Cordum, L. Hillier *et al.*, 2003 The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature **423:** 825-837.

Tajima, K., O. Enishi, M. Amari, M. Mitsumori, H. Kajikawa *et al.*, 2002 PCR detection of DNAs of animal origin in feed by primers based on sequences of short and long interspersed repetitive elements. Biosci Biotechnol Biochem **66:** 2247-2250.

Tilford, C. A., T. Kuroda-Kawaguchi, H. Skaletsky, S. Rozen, L. G. Brown *et al.*, 2001 A physical map of the human Y chromosome. Nature **409:** 943-945.

Tosi, A. J., J. C. Morales and D. J. Melnick, 2000 Comparison of Y Chromosome and mtDNA Phylogenies Leads to Unique Inferences of Macaque Evolutionary History. Mol Phylogenet Evol **17:** 133-144.

Verkaar, E. L. C., I. J. Nijman, M. Beeke, E. Hanekamp and J. A. Lenstra, 2003a Maternal and paternal lineages in cross-breeding bovine species: The unusual phylogeny of wisent. submitted.

Verkaar, E. L. C., I. J. Nijman, K. Boutaga and J. A. Lenstra, 2002 Differentiation of cattle species in beef by PCR-RFLP of mitochondrial and satellite DNA. Meat Science **60:** 365-369.

Verkaar, E. L. C., H. Vervaecke, C. Roden, L. Romero Mendoza, M. W. Barwegen *et al.*, 2003b Paternally inherited markers in bovine hybrid populations. submitted.

Verkaar, E. L. C., C. Zijlstra, R. Jansen, K. Boutaga, G. Arkesteijn *et al.*, 2003c Organisation and concerted evolution of the Y-chromosomal TSPY multigene family in cattle. submitted.

Vogel, T., S. Borgmann, F. Dechend, W. Hecht and J. Schmidtke, 1997a Conserved Y-chromosomal location of TSPY in Bovidae. Chromosome Res **5:** 182-185.

Vogel, T., F. Dechend, E. Manz, C. Jung, S. Jakubiczka *et al.*, 1997b Organization and expression of bovine TSPY. Mamm Genome **8:** 491-496.

Vogel, T., O. Dittrich, Y. Mehraein, F. Dechend, F. Schnieders *et al.*, 1998 Murine and human TSPYL genes: novel members of the TSPY-SET-NAP1L1 family. Cytogenet Cell Genet **81:** 265-270.

Vogel, T., and J. Schmidtke, 1998 Structure and function of TSPY, the Y-chromosome gene coding for the "testis-specific protein". Cytogenet Cell Genet **80:** 209-213.

Will, R. G., 1999 The transmission of prions to humans. Acta Paediatr Suppl **88:** 28-32.

Samenvatting

Het onderzoek beschreven in dit proefschrift is gericht op de structuur en evolutie van het runder Y-chromosoom. In zoogdieren leidt de aanwezigheid van een intact Y-chromosoom tot de mannelijke ontwikkeling. Doorslaggevend is daarbij de aanwezigheid van een intact SRY gen. Recent zijn er ook andere genen geïdentificeerd op het Y-chromosoom en een aantal hiervan spelen een rol bij de spermatogenese. Deleties, amplificaties en translocaties op het Y-chromosoom leiden vaak tot onvruchtbaarheid.

Het **eerste hoofdstuk** is een algemene inleiding. We beginnen met een beschrijving van het humane Y-chromosoom, omdat daarover het meeste bekend is en het model kan staan voor het runder Y-chromosoom. Dit wordt dan in context geplaatst van de bijzondere evolutie van het Y-chromosoom. Het Y-chromosoom is gevoelig voor degeneratie en het is er een komen en gaan van genen die een rol kunnen spelen bij de mannelijke ontwikkeling of vruchtbaarheid. Verder is er een snelle selectie van allelen die mannen meer nakomelingen bezorgen en is het plausibel dat het Y-chromosoom onderhevig is aan een antagonistische evolutie, bijvoorbeeld ten behoeve van de handhaving van de geslachtsverhouding.

Tenslotte besteden wij aandacht aan het Y-chromosoom als merker in de mannelijke lijn voor afstammingsonderzoek ten behoeve van historische studies, forensische analyse of voedselinspectie. Dit wordt besproken samen met het gebruik van andere markers: mitochondriaal DNA als marker van de vrouwelijke lijn en verschillende vormen van variatie in het kern DNA.

Hoofdstuk 2 sluit hier rechtstreeks op aan en bestaat uit drie onderdelen, waarin wij respectievelijk Y-chromosomaal DNA, mitochondriaal DNA, satelliet DNA en SINE elementen gebruiken voor een aantal gerelateerde toepassingen. **Hoofdstuk 2a** beschrijft twee complementaire methoden voor de identificatie van rundvlees. Deze zijn gebaseerd op polymorfismen in respectievelijk satelliet DNA en mitochondriaal DNA. Het satelliet DNA is hierbij vooral relevant voor de identificatie van monsters van hybride oorsprong. **Hoofdstuk 2b** beschrijft het gebruik van Y-chromosomale moleculaire merkers in rundersoorten om mannelijke inkruising aan te tonen. Ter illustratie bestuderen wij de hybride oorsprong van individuele dieren in een Belgische bizon/wisent gemengde popula-

tie en van het Madura veeras in Indonesië. In **hoofdstuk 2c** presenteren wij een real-time PCR test op de aanwezigheid van Bov-A2 SINE elementen. Omdat BSE waarschijnlijk is ontstaan door de besmetting van beendermeel met prionen afkomstig van schapen die aan scrapie leden, is het belangrijk om te kunnen controleren of beendermeel materiaal van herkauwers (rund, schaap, geit maar ook herten) bevat. SINE's zijn gerepeteerde elementen die vaak voorkomen in het genoom van alle herkauwers. De Bov-A2 SINE is homogeen en is daardoor heel geschikt om uiterst kleine hoeveelheden herkauwermateriaal in beendermeel aan te tonen. De hoge gevoeligheid van onze test is bijzonder belangrijk, omdat het DNA in beendermeel gedeeltelijk of geheel wordt afgebroken door de verplichte hittebehandeling.

In **hoofdstuk 3** gebruiken wij segmenten van mitochondriaal en Y-chromosomaal DNA in een fylogenetische studie van de runderachtigen (*Bovini*). Zoals verwacht vonden we een duidelijke verwantschap van taurine vee en zeboe en van gayal, gaur en banteng. Verder is er een duidelijke verwantschap van de yak en de Amerikaanse bizon. De wisent of Europese bizon heeft echter heel anders mitochondriaal DNA dan de Amerikaanse bizon, hoewel beide bizonsoorten volledig kruisbaar zijn zelfs vruchtbare nakomelingen kunnen voortbrengen. De verwantschap van de bizonsoorten komt echter goed tot uiting in het Y-chromosomale DNA. Theoretisch kan het verschil in het mitochondriale DNA van wisent en bison worden verklaard door het naast elkaar voorkomen van twee haplotypen in de bison-wisent-yak lijn, gevolgd door de fixatie van één haplotype in elk van de takken die leiden naar de afzonderlijke soorten (*lineage sorting*). Een meer plausibele mogelijkheid is de introgressie van bizonstieren in een vroege rundachtige populatie, hetgeen zou hebben plaatsgevonden in het late Pleistoceen – vroege Holoceen (10.000 – 20.000 jaar geleden). Dit verklaart de plotselinge opkomst van fossiele overblijfselen van wisent, die niet kunnen worden gerelateerd aan de overblijfselen van andere bizonsoorten. Zo'n mannelijke inkruising zou hebben geleid tot de verdrijving van de oorspronkelijke stieren. Na een aantal generaties van inkruisen is de vruchtbaarheid volledig hersteld en heeft de populatie bizonachtige Y-chromosomen, maar nog steeds de oorspronkelijke mitochondrieën. Volgens de leerboeken kunnen nieuwe soorten ontstaan door 1) geografische barrière (*allopatrese*), 2) differentiatie binnen hetzelfde gebied (*sympatrese*) of 3) afzonderlijke evolutie in aangrenzende gebieden gescheiden door een hybride zone (*parapatrese*). Wij introduce-

ren de term *transpatrese* voor de soortvorming door mannelijke inkruising. Iets vergelijkbaars is al eens waargenomen bij Amerikaanse herten, steenbokken en makaken.

Hoofdstuk 4 beschrijft de organisatie en evolutie van de *TSPY* genen op het Y-chromosoom. *TSPY* lijkt met tweehonderd kopieën een belangrijk gen op het runder Y-chromosoom. In dit hoofdstuk beschrijven wij dat *TSPY* in een aantal verschillende vormen voorkomt: *TSPY-M1* en *TSPY-M2*, allebei een intakt of bijna intakt gen, en *TSPY-C*, een cluster van een gedegenereerde en onvolledig pseudogenen. De TSPY loci bevinden zich voornamelijk op de korte arm van het Y-chromosoom Verder vonden wij in de *TSPY-M* loci *concerted evolution*, omdat de *TSPY* kopieën van het rund meer op elkaar lijken dan op die van andere rundachtigen. Dit toont aan dat de *TSPY* varianten snel kunnen evolueren.

In **hoofdstuk 5** plaatsen we onze resultaten in een breder perspectief en beschrijven de toekomstperspectieven van het onderzoek aan het Y-chromosoom.

List of Publications

1. S.M. Rensen, E.L.C. Verkaar, W.M.H. Debie, G.J.J.M. van Eys, A brief report on the existence of a novel smoothelin isoform, Journal of Molecular Cardiology, 1996 (abstract)

2. G.J.J.M. van Eys, C.J.M. de Vries, S.M. Rensen, V.L.J.L. Thijssen, E.L.C. Verkaar, G.P.G.M. Coolen, W.M.H. Debie, M.C. de Ruiter, S.D. Wadleigh-Detera, Smoothelins: One gene, two proteins, three muscle cell types…so far, Cardiovascular specific gene expression, Ed. by P.A. Doevedans, R.S. Reneman, M. van Bilsen, Kluwer academic publishers, 1999 (Chapter)

3. E.L.C. Verkaar, I.J. Nijman, K. Boutaga, J.A. Lenstra, Differentiation of cattle species in beef by PCR-RFLP of mitochondrial and satellite DNA, Meat Science 60, 365-369, 2002

4. E.C. Soethout, E.L.C. Verkaar, G.H. Jansen, K.E. Muller, J.A. Lenstra, A direct StyI polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) test for the myophosphorylase mutation in cattle, Journal of Veterinary Medicine A, 49, 1-2, 2002

5. J. Neves, M. Abecassis, T. Santiago, T. Ramos, J. Melo, E. Gruys, K. Hulskamp-Koch, A. Ultee, E.L.C. Verkaar, J.A. Lenstra, Y.A. Goffin, A. Vanderkelen, B. van Hoeck, C.J. Hunt, D.E. Pegg, Processing of ovine cardiac valve allografts: 3. Implantation following antimicrobial treatment and preservation, Cell and Tissue Banking, 3, 105-119, 2002

6. I.J. Nijman, M. Otsen, E.L.C. Verkaar, C. de Ruijter, E. Hanekamp, J.W. Ochieng, S.B.M. Shamshad, E.O. Regge, O. Hanotte, J.A. Lenstra, Hybridisation of banteng (Bos javanicus) and zebu (Bos indicus) revealed by mitochondrial DNA, satellite DNA, AFLP and microsatellites, Heredity, Jan; 90(1): 10-16, 2003

7. E.L.C. Verkaar, H. Vervaecke, C. Roden, L. Romero-Mendoza, M.W. Barwegen, T. Susilawati, I.J. Nijman, J.A. Lenstra, Paternally inherited markers in bovine hybrid populations, Heredity in Press

8. L. Romero-Mendoza, E.L.C. Verkaar, P.J.M. Savelkoul, A. Catsburg, H.J.M. Aarts, J.B. Buntjer, J.A. Lenstra, Sensitive and semi-quantitative detection of ruminant DNA, Journal of Food Protection in Press

9. E.L.C. Verkaar, I.J. Nijman, M. Beeke, E. Hanekamp, J.A. Lenstra, Maternal and paternal lineages in cross-breeding bovine species: The unusual phylogeny of wisent, submitted

10. E.L.C. Verkaar, C. Zijlstra, E.M. van 't Veld, K. Boutaga, D.C.J. van Boxtel, J.A. Lenstra, Organization and concerted evolution of the ampliconic Y-chromosomal TSPY gene of cattle, in preparation

Curriculum vitae

Edward Louis Christian Verkaar werd geboren op 26 juli 1969 te Leiden. In 1989 haalde hij het HAVO diploma en in 1991 het VWO diploma. Een opleiding tot beroepsvlieger kon niet worden afgemaakt vanwege een oogzenuwontsteking waarna tot 1993 een universitaire opleiding Geneeskunde volgde in Antwerpen en tot 1998 Gezondheidswetenschappen in Maastricht. In die periode heeft hij stages vervuld aan de afdelingen Microbiologie en Moleculaire Celbiologie. Vanaf 2 februari 1999 was hij werkzaam als assistent in opleiding aan de afdeling Bacteriologie, Universiteit Utrecht en werd het werk zoals beschreven in dit proefschrift uitgevoerd.

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